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# Synthesis of 1,3-Oxazine derivative from Chalcone and Screening for their Anti-Oxidant and Anti-Inflammatory activity

**Chaitra G*and Rohini RM**

Department of Pharmaceutical Chemistry, Al-Ameen College of Pharmacy, Hosur road, near Lalbhag, Bengaluru-560 027

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***Corresponding author:**
**chai238narasaiah@gmail.com**



## Abstract

Oxazine heterocycles have special interest due to its important class of natural and non- natural products and show high biological activities in pharmaceutical and biological fields. Research work was planned to synthesize 1,3-oxazine derivative from chalcone and screened for their anti-inflammatory and anti-oxidant activity. The structure of synthesized compounds 5(a-f) were established from UV, IR, $^{1}$H NMR, $^{13}$C NMR and Mass spectral data. All the synthesized compounds were screened for *in-vitro* anti-inflammatory by bovine serum albumin and protease method and *in-vitro* anti-oxidant by diphenyl picryl hydrazide and nitric oxide method. Among synthesized derivatives, the screening results revealed 5(c) and 5(e) to be significant at concentration of 10 µg /ml.

**Key words:** chalcones, oxazine, anti-inflammatory activity, anti-oxidant activity.

## INTRODUCTION

Heterocyclic compounds are basis for many pharmaceutical, veterinary and agrochemical products [1]. Synthesis of oxazine was main interest as it is one of the important compound of heterocycle encompassing nitrogen and oxygen atom in six membered aromatic ring. It exist in three isomeric forms depending on its relative position of the heteroatom's and relative position of the double bonds. Isomeric oxazine derivatives synthesized from chalcone are known to possess various activities like anti- hyperglycemic [2], anti-microbial [3], anti-ulcer [4], anti-inflammatory [5], anti-malarial [5], analgesic [6], anti-cancer [7], anti-tubercular [8], anti-oxidant [9], anti-viral [10] and anti-leishmanial [11], anti-coagulant activities [22,23]. Oxazine derivatives have played very important role in the improvement of heterocyclic

chemistry and ordinarily used in organic synthesis [13]. Oxazine derivatives have been reported to possess anti-fungal [14,15,16,17,18], antibacterial [19,20,21], Due to its numerous biological activities reported , we planned to synthesis 1,3-oxazine derivatives in our research and carry out the *in vitro* anti-inflammatory and anti-oxidant activities .

**MATERIALS AND METHODS**

All the reagents and solvents used were of laboratory grade. The melting points of synthesized compounds were determined by Electro Thermal apparatus using fused capillary tubes expressed in °C. Monitoring the reaction and purity of the compounds was checked by thin-layer chromatography (TLC) using silica gel G plates of 0.5 mm thickness as stationary phase and combination of n-hexane: ethyl acetate in different ratios as mobile phase. The spots were visualized by using iodine chamber and UV chamber. The UV spectra of the synthesized compounds were recorded on Shimadzu UV-1601 and the values of wavelength (λ-max) were reported in nm. The IR spectra of the compounds were recorded on a Fourier Transform IR spectrophotometer (model Shimadzu 8700) in the range of 4000-400 $cm^{-1}$ using KBr pellets and value of λ-max are reported in $cm^{-1}$ and the spectra were interpreted. $^{1}$H-NMR and $^{13}$C-NMR spectra were recorded on Bruker Avance II 400 NMR spectrometer using $CDCl_3$. Chemical shift (δ) are reported in parts per million downfield using internal reference Tetra methyl silane (TMS) and the spectra were interpreted. Mass spectra were recorded on Mass spectrophotometer (model Shimadzu) by MS-2010A.

**Synthesis of N-(4-Acetyl-phenyl)-isonicotinamide (03)**

To an ice cold solution of isonicotinyl chloride (0.01 mol) dissolved in methanol, a methanolic solution of *p*-amino acetophenone (0.01 mol) was added dropwise with constant stirring. Stirring was continued for 15 mins then refluxed for one hour. The reaction mixture was cooled and the obtained precipitate was filtered. Crude product was recrystallized from isopropyl alcohol.

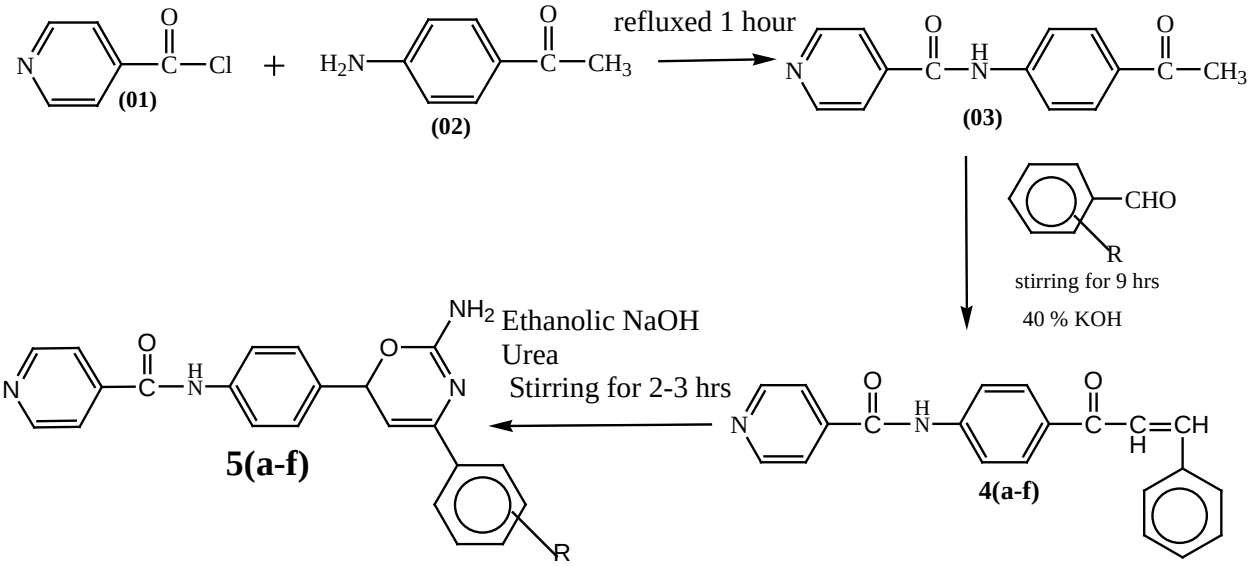
**Synthesis of chalcones 4(a-f)**

Equimolar quantity of *N*-(4-acetylphenyl)-isonicotinamide (0.01 mol) and substituted benzaldehyde (0.01 mol) were dissolved in absolute alcohol and 40 % KOH solution was added slowly with stirring the reaction was continued for 9 hours and left overnight. The reaction mixture was decomposed in ice-water to obtain product. Crude product was recrystallized from ethanol.

**Synthesis of oxazine derivatives 5(a-f)**

Equimolar quantity of **4 (a-f)** and urea were dissolved in ethanolic NaOH and was stirred for 2-3 hours at room temperature. Refluxed for 6 hours and poured into cold water with continuous stirring for one hour and was cooled at 0 $^{o}$ C for 42 hours. The precipitate obtained was filtered, washed and re-crystalized from ethanol.

**Scheme of synthesis**




***In-vitro* anti-inflammatory Bovine serum albumin (BSA) activity**

A solution of 0.2 % w/v of BSA was prepared in Tris buffer saline. Stock solutions 1000 µg /ml of compounds to be tested were prepared, two different concentrations of 100 µg /ml and 200 µg /ml were prepared by using methanol as a solvent. To each test sample (0.1 ml), 5 ml of 0.2 % BSA was added. The blank consisted 5 ml 0.2 % w/v BSA solution and 0.1 ml methanol. The positive control consist 0.1ml (100 µg/ml) of indomethacin in methanol and 5 ml 0.2 % w/v BSA solution. The test samples were heated at 72 ˚ C for five minutes and then cooled for 10 min. The absorbance of these solutions was determined by using spectrophotometer at a wavelength of 660 nm. The denaturation of the protein was determined on a percentage basis relative to the blank control using the following formula:-

$$\% \text{ inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

**Protease inhibition assay method**

The reaction mixture was containing 0.5 ml of protease solution (0.06 mg of trypsin in 20 mM of Tries HCl buffer), 0.5 ml of Tris HCl buffer (20 mM) and 1 ml of test sample of different concentration. The mixture was incubated at 37 $^{o}$ C for 15 min and 1 ml of 0.8 % w/v casein was added. The mixture was incubated for an additional 20 min. Then 2 ml of 70 % perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The % inhibition was calculated.

$$\% \text{ inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

***In-vitro* antioxidant Diphenyl picryl hydrazide (DPPH) activity**

A stock solution of DPPH (10 mg) was prepared by dissolving in 10 ml of methanol. From this stock solution, different dilutions were made to obtain concentrations of 10 to 100 µg/ml. The absorbance was recorded at 517 nm.

Standard solution ascorbic acid (10 mg) was dissolved in 10 ml of methanol. From this stock solution dilutions 10 to 100 µg/ml were measured. From each concentration of ascorbic acid solutions 1 ml was taken into 1 ml of DPPH solution of 30 µg/ml concentration and volume was made up to 10 ml with methanol. The absorbance was recorded for these dilutions at 517 nm after incubationfor30mins.The effect of ascorbic acid (vitamin C) on DPPH was assessed for compar ison with that of synthesized compounds.

The test samples were prepared by initial preparation of stock solution of (1000 µg/ml) in methanol and from this various concentrations were prepared (100, 50 and 10 µg/ml). To all these dilutions, 1 ml of DPPH solution (30 µg/ml) was added and then absorbance were recorded at 517 nm after duration of 30 min. Percentage inhibition of free radical activity was calculated using the following formula.

$$\% \text{ inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

**Nitric oxide radical scavenging method**

To 0.5 ml of 10 mM sodium nitroprusside (10mM) in phosphate buffer saline was added to 1 ml of test sample of different concentration, incubated at 25 $^{o}$ C for 180 min then added 1ml of griess reagent [Solution A: 1 % sulphanilamide in 2.5 % phosphoric acid; Solution B: 0.1 % napthyl ethylene diamine in 2.5 % phosphoric acid]. Solution A and solution B were mixed in equal volume and were used before 12 hrs. The positive control ascorbic acid solution was prepared similarly and was incubated. A blank solution was prepared. The absorbance of chromophore formed was measured at 546 nm on UV-Visible.

$$\text{NO Scavenging (\%)} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

**RESULTS AND DISCUSSION**

The physical properties of the synthesized compounds are shown in table no: 01

**Table-01: Physical data of 1,3-oxazine derivatives**

| Sl.No | Compound | R | Mol.Formula | Mol.Weight | M.P in $^oC$ | % yield |
|---|---|---|---|---|---|---|
| 1 | 5(a) | H | $C_{23}H_{23}N_4O_2$ | 387.45 | 108$^o$ C | 65.48 |
| 2 | 5(b) | $CH_3$ | $C_{23}H_{23}N_4O_2$ | 400.47 | 87$^o$ C | 92.33 |
| 3 | 5(c) | $(OCH_3)_3$ | $C_{26}H_{28}N_4O_5$ | 476.52 | 154$^o$ C | 98.2 |
| 4 | 5(d) | Cl | $C_{26}H_{28}N_4O_5$ | 420.89 | 180$^o$ C | 70.0 |
| 5 | 5(e) | $NO_2$ | $C_{23}H_{21}N_5O_4$ | 431.44 | 158$^o$ C | 72.4 |
| 6 | 5(f) | $N(CH_3)_2$ | $C_{25}H_{27}N_5O_4$ | 429.51 | 190$^o$ C | 75.8 |

**Compound 5(a)**

IR (KBR, $cm^{-1}$): 3462.56 $cm^{-1}$($NH_2$), 3359.39 $cm^{-1}$ NH, 3052.76 $cm^{-1}$ C-H (Ar), 1120 $cm^{-1}$ C-O-C, 1451.17 $cm^{-1}$ C=C (Ar), 1563.88 $cm^{-1}$ CONH.

$^1$H NMR ($CDCl_3$-ppm): δ 7.96 (d,1H,CONH), δ 7.57 (d,2H,ArH), δ 7.17 (m,1H,ArH), δ 7.30 (d,2H,ArH), δ 7.21 (s,2H,ArH), δ 2.0 (s,2H,$NH_2$), δ 6.7 (d,1H,C=C), δ 5.19 (d, 1H, CH oxazine), δ 5.19 (d,1H,C-O), δ 7.96 (m, 4H, 4-pyridine), δ 7.17 (m, 4H, 1-benzene) δ 2.0 (d, 2H, $NH_2$).

**Compound 5(b)**

IR (KBR, $cm^{-1}$): 3462 $cm^{-1}$ ($NH_2$), 3341.07 $cm^{-1}$ (NH), 3046.98 $cm^{-1}$ (Ar C-H), 1120 $cm^{-1}$ (C-O-C), 1438.6 $cm^{-1}$ (Ar C=C), 1511.92 $cm^{-1}$ (CONH), 1611.92 $cm^{-1}$ (C=N).

$^1$H NMR ($CDCl_3$-ppm): δ 7.93 (d,1H,CONH), δ 7.51 (d,2H,ArH), δ 7.2 (m,1H,ArH), δ 7.4 (d,2H,ArH), δ 7.29 (s,2H,ArH), δ 2.40 (s,2H,$NH_2$), δ 3.73 (d,3H,$CH_3$), δ 6.69 (d,1H,C=C), δ 5.2 (d, 1H, CH oxazine), δ 6.68 (d,3H,C-O).

**Compound 5(c)**

IR(KBr)($cm^{-1}$): 3444.24 $cm^{-1}$ ($NH_2$), 3341.32 $cm^{-1}$ (NH), 3058 $cm^{-1}$ ( Ar C-H), 3003 $cm^{-1}$ (C-O-C) 1058 $cm^{-1}$ (Ar C=C) 1504&1417 $cm^{-1}$, (CONH) 1584 $cm^{-1}$, (C=N) 1634 $cm^{-1}$.

$^1$H NMR ($CDCl_3$-ppm): δ 7.93 (d,1H,CONH), δ 7.91 (d,2H,ArH), δ 7.7 (m,1H,ArH), δ 7.4 (d,2H,ArH), δ 7.29 (s,2H,ArH), δ 4.40 (s,2H,$NH_2$), δ 3.8 (d,9H,$OCH_3$), δ 6.69 (d,1H,C=C), δ 5.2 (d, 1H, CH oxazine), δ 6.68 (d,3H,C-O).

$^{13}$C NMR ($CDCl_3$-ppm): (2C,-C=N) δ 151.57, (4C,-C=C) δ 128.12, (1C,-CONH) δ 188.02, (2C,ArH) δ 139.91, (2C,ArH) δ 121.35, (1C,-C-O) δ 60.98, (1C,$NH_2$) δ 153.39, (1C,-C-C) δ 113.87, (1C,-C-N) δ 130.87, (1C,ArH) δ 143.25, (2C,ArH) δ 105.42, (3C,ArH) δ 131.11, (3C,$OCH_3$) δ 56.19.

MS *(m/z):* M+ calculated 476.52, found 476.0.

**Compound 5(d)**

IR(KBr)($cm^{-1}$): 3425.92 $cm^{-1}$ ($NH_2$), 3333.36 $cm^{-1}$ (NH), 3058 & 3003 $cm^{-1}$ (Ar C-H), 1178.29 $cm^{-1}$ (C-O-C), 1442.49 $cm^{-1}$ (Ar C=C), 1579.41 $cm^{-1}$ (CONH), 1652.7 $cm^{-1}$ (C=N).

$^1$H NMR ($CDCl_3$-ppm): δ 7.81 (d,1H,CONH), δ 7.52 (d,2H,ArH), δ 7.2 (m,1H,ArH), δ 7.4 (d,2H,ArH), δ 7.3 (s,2H,ArH), δ 2.0 (s,2H,$NH_2$), δ 6.69 (d,1H,C=C), δ 5.1 (d, 1H, CH oxazine), δ 6.68 (d,3H,C-O).

**Compound 5(e)**

IR(KBr)($cm^{-1}$): 3474.13 $cm^{-1}$ ($NH_2$), 3359.39 $cm^{-1}$ (NH), 3076 $cm^{-1}$ (Ar C-H), 1178.29 $cm^{-1}$ (C-O-C),1438.64 $cm^{-1}$ (Ar C=C), 1500.39 $cm^{-1}$ (CONH), 1592.29 $cm^{-1}$ (C=N).

$^1$H NMR ($CDCl_3$-ppm): δ 7.83 (d,1H,CONH), δ 7.59 (d,2H,ArH), δ 7.31 (m,1H,ArH), δ 7.54 (d,2H,ArH), δ 7.29 (s,2H,ArH), δ 2.20 (s,2H,$NH_2$), δ 6.69 (d,1H,C=C), δ 5.2 (d, 1H, CH oxazine), δ 6.68 (d,3H,C-O).

**Compound 5(f)**

IR(KBr)($cm^{-1}$): 3437.49 $cm^{-1}$ ($NH_2$), 3351.68 $cm^{-1}$ (NH), 3030.18 $cm^{-1}$ (Ar C-H), 1069.33 $cm^{-1}$ (C-O-C), 1590.02&1432.85 $cm^{-1}$ (Ar C=C), 1524.45 $cm^{-1}$ (CONH), 1662.34 $cm^{-1}$ (C=N).

$^1$H NMR ($CDCl_3$-ppm): δ 7.90 (d,1H,CONH), δ 7.50 (d,2H,ArH), δ 7.29 (m,1H,ArH), δ 7.4 (d,2H,ArH), δ 7.32 (s,2H,ArH), δ 2.0 (s,2H,$NH_2$), δ 6.69 (d,1H,C=C), δ 5.19 (d, 1H, CH oxazine), δ 6.68 (d,3H,C-O), δ 2.85 [m,6H, $N(CH_3)_2$].

***In-vitro* anti-inflammatory activity method by BSA**

*In vitro* for anti-inflammatory activity by BSA method are shown in table no-02, all compounds have shown dose dependent significant effect and compound **5(c)** and **5(e)** have shown very significant effect .

**Table-02: Anti-inflammatory activity of oxazine derivatives (BSA method)**

| **Anti-inflammatory activity by BSA method** | | |
|---|---|---|
| **Compound code** | **Percentage Inhibition** | |
| | **Dose (100µg/ml)** | **Dose (200µg/ml)** |
| Indomethacin | 78.01 | 94.8 |
| 5a | 66.6 | 73.3 |
| 5b | 66.6 | 86.6 |
| 5c | 66.6 | 86.6 |
| 5d | 40.0 | 46.6 |
| 5e | 66.6 | 80.0 |
| 5f | 66.6 | 86.6 |

**Proteases method**

All the synthesized compounds were screened Proteases method for *in-vitro* anti-inflammatory activity (table no -03). It was evident by the observation that the compound **5(c)** and **5(e)** exhibited excellent anti-inflammatory activity.

**Table-03: Anti-inflammatory activity (proteases method)**

| Anti-inflammatory activity by Protease method | | | |
|---|---|---|---|
| **Compound code** | **Percentage inhibition** | | |
| | **Dose (100 µg/ml)** | **Dose (50 µg/ml)** | **Dose (10µg/ml)** |
| Indomethacin | 89.32 | 69.31 | 30.15 |
| 5a | 70.92 | 67.97 | 61.29 |
| 5b | 76.06 | 48.38 | 27.86 |
| 5c | 76.85 | 67.46 | 51.33 |
| 5d | 71.76 | 68.06 | 48.48 |
| 5e | 84.29 | 73.77 | 56.66 |
| 5f | 80.64 | 62.13 | 82.14 |

**Anti-oxidant activity by DPPH method**

DPPH radical scavenging assay is the most widely used method for antioxidant activity due to hydrogen donation. It can be visually observed as the color changes from purple to yellow. **5(c)** and **5(e)** synthesized compounds exhibited good activity as compared to standard ascorbic acid. The percentage inhibition of free radical is shown in table-04

**Table-04: Anti-oxidant activity DPPH method**

| Anti-oxidant activity by DPPH method | | | |
|---|---|---|---|
| **Compound code** | **Percentage inhibition** | | |
| | **Dose (100 µg/ml)** | **Dose (50 µg/ml)** | **Dose (10 µg/ml)** |
| Ascorbic acid | 99.25 | 95.41 | 90.5 |
| 5a | 94.4 | 94.0 | 93.5 |
| 5b | 89.5 | 89.4 | 89.0 |
| 5c | 84.7 | 82.8 | 82.3 |
| 5d | 88.4 | 88.9 | 87.5 |
| 5e | 93.25 | 89.0 | 88.5 |
| 5f | 88.0 | 64.0 | 63.0 |

**Nitric oxide (NO) method:-**

In nitric oxide radical scavenging activity the compounds decompose in aqueous solution at physiological pH (7.2) which produces NO under aerobic conditions in the presence of sodium nitroprusside. No reacts with oxygen to produce nitrate and nitrite. The reduction of nitrite concentration in the assay media is measured by griess reagent.

Compounds 5(c) and 5(e) exhibited potent anti-oxidant activity. The % percentage inhibition are depicted in the table no: 05

**Table-05: Anti-oxidant activity by NO method**

| Anti-oxidant activity by NO method | | | |
|---|---|---|---|
| Compound code | Percentage inhibition | | |
| | Dose (100 µg/ml) | Dose (50 µg/ml) | Dose (10 µg/ml) |
| Ascorbic acid | 81.112 | 80.4 | 78.81 |
| 5a | 80.48 | 80.48 | 80.48 |
| 5b | 81.11 | 81.73 | 81.11 |
| 5c | 81.11 | 81.11 | 81.73 |
| 5d | 89.48 | 85.73 | 81.11 |
| 5e | 80.48 | 81.11 | 81.11 |
| 5f | 80.48 | 82.32 | 80.48 |

## CONCLUSION

The results of the study of synthesized novel 1,3-oxazine substituted derivatives showed moderate to good yield that exhibited satisfactory *in-vitro* activity. We can conclude that oxazine derivatives showed promising anti-oxidant anti-inflammatory activity. The most potent anti-oxidant anti-inflammatory compound was found to be 5(c) and 5(e).

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