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In vitro anti-cancer, anti-inflammatory and anti-oxidant studies of novel oxazine derivatives

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Abstract
The ability to inhibit oxidative stress has been established as the prime mechanism in treatment of several disease conditions. Condensation of substituted acetophenones and Ferrocene aldehyde resulted in the formation of various Chalcones (I-III). The resulting various Chalcones (I-III) react with urea to form various oxazines ((Ia-IIIa). The reaction was monitored by TLC and synthesized products were characterized by physical, Elemental analysis, Spectral studies UV, FT-IR, C^{13} and ^{1}H-NMR Spectroscopy. When these compounds were screened for antioxidant susceptibilities by FRAPAssay, anti-inflammatory and anti-cancer activities.

Keywords: Ferrocene aldehyde, substituted acetophenones, chalcones, oxazines, thiazines

Introduction
Many present day diseases are reported to be due to an impaired balance of the pro-oxidant - antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either on account of increased generation of free radicals caused by excessive oxidative stress, or due to poor scavenging in the body caused by depletion of the dietary antioxidants. Reactive oxygen species differ significantly in their interactions and can cause extensive cellular damage such as nucleic acid strand scission, modification of polypeptides, lipid peroxidation etc. [1]. There is increasing experimental, clinical and epidemiological evidence highlighting an involvement of free radicals and reactive oxygen species (ROS) in a variety of human diseases including cancer, inflammatory disorders and various degenerative ailments associated with aging [2]. Antioxidants are chemical substances, which scavenging free radicals and ROS thereby minimizing the burden of oxidative stress generated in the body [3]. Interestingly, antioxidants can protect critical cell macromolecules (proteins, lipids & nucleic acids) from undergoing oxidation and thus help in health amelioration [4]. Antioxidants can be defined as "any substance which significantly delays or inhibits oxidative damage to a target molecule". Antioxidants are the first line of defense against free radical damage, and are critical for maintaining optimum health. The need for antioxidants becomes even more critical with increased exposure to free radicals. As part of a healthy lifestyle and a well-balanced, wholesome diet, antioxidant supplementation is now being recognized as an important means of improving free radical protection [5].
In the present study we have undertaken the evaluation of antioxidant, anti-inflammatory and anti-cancer activity of novel oxazines obtained from various chalcones which are well known for their antioxidant activity. Antioxidants are compounds capable of preventing the damage caused in human tissue by the normal effects of physiological oxidation. Research has shown that antioxidants can play a role in preventing the development of chronic diseases such as cancer, diabetes and cardiovascular diseases [6]. Heterocyclic compounds particulary five or six member ring compounds have occupied the first place among various classes of organic compounds for their diverse biological activities. The heterocyclic chemistry is composed of 5-membered, 6-membered and fused heterocycles [7]. These compounds posses one or the other chemotherapeutic or pharmacological activities [8]. It is commonly reported that heterocycles having sulphur or nitrogen atoms or both of them are the general features present in the structures of most of the pharmaceutical and natural compounds [9]. Organometallic chemistry is growing interest especially in the recent decades due to its wide applications in the biological and medicinal field, this application leads to a new area called bioorganometallic chemistry [10]. Ferrocene moiety is used in bioorganometallic chemistry due to its stability, biological activity and application in organic synthesis to prepare new compounds [11].

Decoding the action mechanism of ferrocene based drugs reveal an indispensable role of the metallocene moiety in drug action thus widening the scope of bioorganometallic chemistry for the next generation drug discovery [12]. Chalcones are prepared by condensing Aryl ketones with aromatic aldehydes in presence of suitable condensing agents [13]. The compounds with the backbone of chalcones have been reported to exhibit a wide variety of pharmacological activity including antimalarial [14], antibacterial [15], antitumor [16], anticancer [17], anti-inflammatory [18], antifungal [19], antioxidant [20], antileishmanial [21].

Experimental

The synthesized test compounds were purified by recrystallization and characterized by UV-Visible, Infra Red, [1] H and [13] C NMR spectroscopy.

According to A. Suyambulingam, S. Nair and K. Chellapandian is concerned withthe Synthesis of chalcones (I, II, III), oxazines (Ia, IIa, IIIa), spectral properties, antibacterial and antifungal studies [22].

General procedure

Synthesis of Chalcones [23] (I-III)

Equimolar amounts of Ferrocene aldehyde (0.01mol) and 4-cholro acetophenone, 4-methoxy acetophenone/4-piperazine acetophenone (0.01mol) were dissolved in ethanol (25 mL), 10% NaOH solution was slowly added, and the mixture was stirred for 4 hours before being poured into 400 ml of water with constant stirring and left overnight in the refrigerator. The filtered precipitate was washed with distilled water until it was free of NaOH and recrystallized from ethanol. TLC was used to examine the data, and melting points were measured using a digital melting point apparatus.

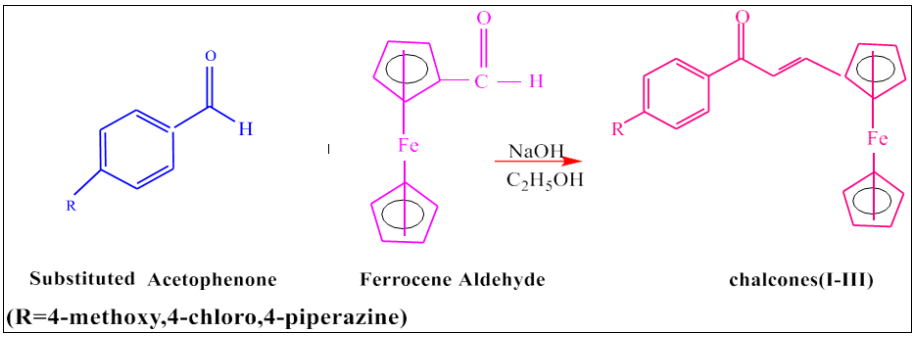

Fig 1: Synthetic scheme for the synthesis of Chalcones (I-III)

Preparation of oxazine deriavatives (Ia–IIIa)

A magnetic stirrer was used to stir a combination of chalcones (I-III) (0.02 mol) and Urea (0.02 mol) dissolved in an ethanolic sodium hydroxide (30 mL) for around 3 hours. It was then placed into 400 mL of cold water and stirred continuously for an hour before being chilled for 24 hours. From ethanol, the precipitate was filtered, washed, and recrystallized. TLC was used to examine the data, and melting points were measured using a digital melting point apparatus

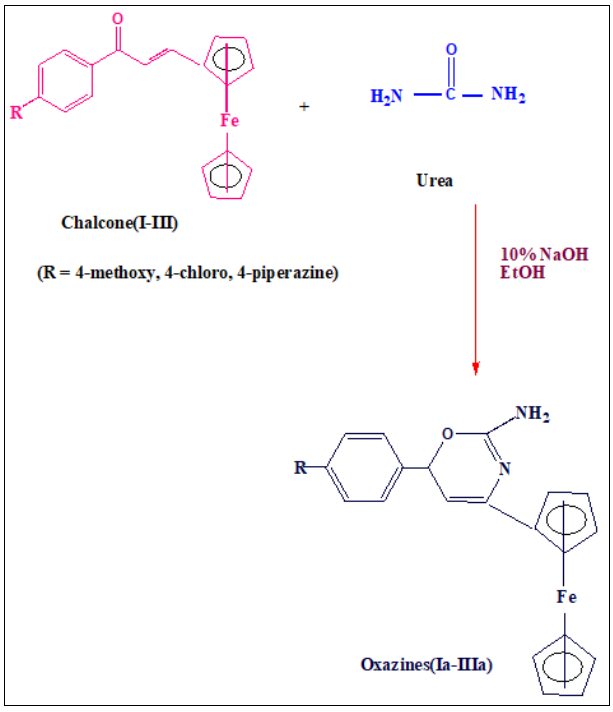

Fig 2: Synthetic scheme for the synthesis of Oxazines (I-III)

Antioxidant activity

Ferric Reducing Antioxidant Potential (FRAP) Assay

The ferric reducing power of plant extracts was determined using a modified version of the FRAP assay [24]. This method is based on the reduction, at low pH, of a colorless ferric complex (Fe^{3+}-tripyridyltriazine) to a blue-colored ferrous complex (Fe^{2+}- tripyridyltriazine) by the action of electron-donating antioxidants. The reduction is monitored by measuring the change of absorbance at 593 nm. The working FRAP reagent was prepared daily by mixing 10 volumes of 300 mM acetate buffer, pH 3.6, with 1 volume of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM hydrochloric acid and with 1 volume of 20 mM ferric chloride. A standard curve was prepared using various concentrations of $FeSO_4 \times 7H_2O$. All solutions were used on the day of preparation. One hundred microliters of sample solutions and 300 µL of deionized water were added to 3 mL of freshly prepared FRAP reagent.

The reaction mixture was incubated for 30 min at 37 °C in a water bath. Then, the absorbance of the samples was measured at 593 nm. A sample blank reading using acetate buffer was also taken. The difference between sample absorbance and blank absorbance was calculated and used to calculate the FRAP value. In this assay, the reducing capacity of the plant extracts tested was calculated with reference to the reaction signal given by a Fe^{2+} solution. FRAP values were expressed as mmol Fe^{2+}/g of sample. All measurements were done in triplicate.

Anti-inflammatory assays

Cyclooxygenase (COX) activity

The COX activity was assayed by the method of Walker and Gierse [25]. 100µl cell lysate was incubated with Tris-HCl buffer (pH 8), glutathione 5 mM/L, and hemoglobin 5 mM/L

for 1 minute at 25 °C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes incubation at 37 °C, by the addition 200µL of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 200µL of 1% thiobarbiturate, the tubes were boiled for 20 minutes. After cooling, the tubes were centrifuged for three minutes. COX activity was determined by reading absorbance at 632 nm.
Percentage inhibition of the enzyme was calculated as,

% inhibition = ((Absorbance of control-Absorbance of test)/Absorbance of control) × 100

Lipoxygenase (LOX) activity
The determination of LOX activity was done as per Axelrod *et al*. [26]. The reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The LOX activity was monitored as an increase of absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid.

Percentage inhibition of the enzyme was calculated as,
% inhibition = ((Absorbance of control-Absorbance of test)/Absorbance of control) × 100

Anti-cancer assay
The anticancer properties of the synthesized oxazine(IIa) derivative were tested using the MTT assay method on a number of different cancer cell lines (MCF-7) [23]. Without resorting to the use of radioactivity, the MTT approach provides a fast and simple method for determining whether cells are actively dividing and developing. Cells that had reached confluence were seeded in 96-well plates using DMEM high glucose after being transferred from flasks. the appropriate cell density was maintained (20,000 cells per well). Warmth was maintained for a whole day on the plates by maintaining a temperature of 37 degrees Celsius. The cancer cells were cultivated for 24 hours at 37 °C in an environment containing 5% CO2 after being treated with DMSO (for the positive control group) and the chemical oxazine (IIa) at various doses (12.5, 25, 50, 100, and 200 g/ml) in DMSO. The culture was carried out in an environment with 5% CO2 in the air. From 12.5 to 200 g/ml were the different concentrations. The treatment for the positive control group was the injection of DMSO. The MTT solution, which had a concentration of 0.5 mg/mL, was added, and the plates were then incubated for three hours. The concentration of the MTT solution was determined by the results of the previous step. The optical densities of the DMSO and celss were then measured using an ELISA reader calibrated to 570 nm. Reading the control wells, where it was presumed that the substance's concentration was 100%, yielded the average absorbance readings. The absorbance readings from the wells after the addition of the chemical oxazine (IIa) and the solvent DMSO were used to compute the percentages of cell viability. These absorbance readings were utilized in the calculation. You may use the equation below as a guide to determine whether or not a cell is viable by looking at its ability to reproduced.

% cell viability = [Mean abs of treated cells/Mean abs of Untreated cells] x 100

Result and Discussion
All the synthesized compounds were characterized by TLC, Melting point, elemental analysis, UV-Visible, IR and ^{1}H, ^{13}C NMR Spectroscopy. Analysis indicated by the symbols of the elements is very close to the theorectical values. The compounds were evaluated for Antioxidant activity. The results of antioxidant values expressed as IC_{50} with Ferric Reducing Antioxidant Potential Assay shown in Table.1.Out of three compounds test using FRAP method, Compounds IIIa, Ia, IIa showed IC_{50} values at 165.08,193.83 and 277.927µM respectively when compared to that of the standard ascorbic acid at 518.10µM.The oxazine, which include strong electron-withdrawing substituents such as phenylpiperazinepharmacophorespecificallyattheC-4 position of aromaticring-B, demonstrated good antioxidant properties.

Table 1: Comparison of the IC_{50} of test compounds Against FRAP free radical

Compounds	FRAP Free Radical IC_{50}(µM)
Ia	193.83
IIa	277.92
IIIa	165.08
Standard(Ascorbic acid)	518.10

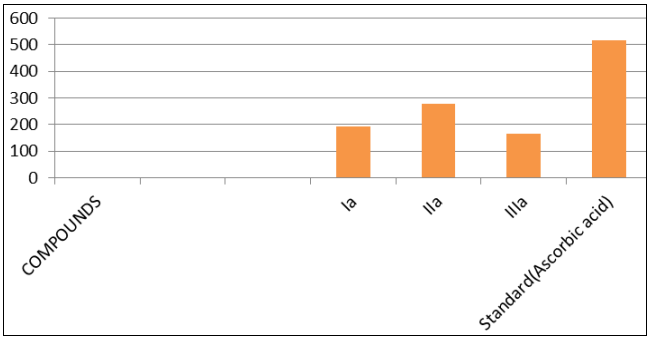

Fig 3: Comparison of the IC50 of test compounds Against FRAP free radical

Anti-inflammatory Assay
Only compounds (Ia), (IIa), and (IIIa) containing a chemical that has substantial antioxidant activity were evaluated for their effectiveness against inflammation in this investigation. The ability of the synthetic thiazine to reduce inflammation was examined using tests for the COX and LOX enzymes. Compound (IIa) revealed an IC50 value for cyclooxygenase (COX) activity at 76.10 M, which was much higher than the reference diclofenac, which was measured at 42.38 M. Compound (IIa) demonstrated lipoxygenase (LOX) activity with an IC50 value of 88.08 M, which is much higher than that of the standard diclofenac, which has an IC50 value of 45.85 M. It was discovered that the compound with the aromatic ring chlorosubstituted (IIa), which demonstrated greater inhibition, had this substitution.

Anticaner Assay
According to the results of the *in vitro* anticancer studies, the chemical with the formula IIa had increased anticancer activity against the MCF7 (human breast) cell. The IC50 value for this chemical was 192.04 M. These results show that the suppression of the proliferation of cancer cells was significantly aided by the presence of both an amino group attached to the aromatic ring B's 2' position as well as a group attached to the aromatic ring B's C-4 position. It was also shown that the presence of both of these groups had a positive synergistic effect on the circumstance. The ant proliferative effect of the compounds that were created was discovered to be greatest in the compounds (IIa) that had an amino group as

an attached component. As a result of these findings, we may deduce that the amino group has a very little effect on the antiproliferative function. Figure 4 depicts the percentage of MCF-7 cells that are still alive after being exposed to several doses of the chemical oxazine (IIa).

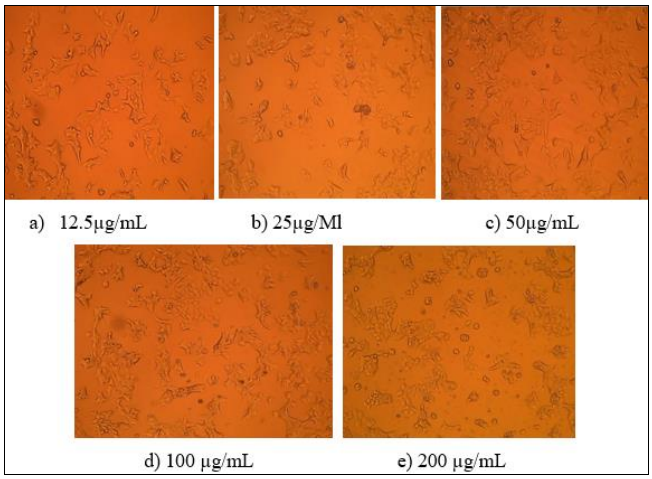

Fig 4: The relative cell viability (%) of MCF-7 cells following the exposure of various concentrations of oxazine(IIa) compound

Conclusion

The antioxidant properties of the synthesized test compounds of oxaines when tested using FRAP assay, the compound IIIa, oxazine gave better antioxidant activity for both oxazines (Ia) and (IIIa). However, theevaluation *in vitro* method showed that the test compounds such as Ia, IIIa had maximum antioxidant properties. The compounds (IIa) were shown to have a gentler effect compared to the effects of conventional diclofenac, COX, and LOX. The MTTassay was used to evaluate a novel oxazine chemical (IIa) to see if it may inhibit the growth of cancer cells *in vitro*. According to these findings, chemical (IIa) is more effective at battling cancers.

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