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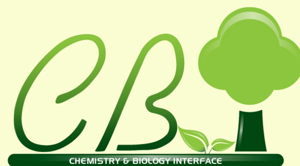

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DESIGN SYNTHESIS OF SOME NOVEL IMINES AND AMIDES HAVING ANTIOXIDANT ACTIVITY

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Abstract: Oxidative stress is one of the major factors contributing to the high morbidity and mortality rates associated with several diseases. Reactive oxidants including reactive oxygen species and reactive nitrogen species are the major contributors of oxidative stress. Therefore, antioxidant supplementation is essential and often required to prevent reactive oxidant-induced pathologies. The aim of the present work is to synthesize a series of imine and amide molecules with putative antioxidant activities so that they can be used in future for new-generation antioxidant drug development. The antioxidant activity was evaluated by Ferric Reducing Antioxidant Power (FRAP) and 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) free radical scavenging assay.

Keywords: Antioxidant, Amides, Imines, Reactive oxidants, Free radicals

Introduction

Oxidative stress is a pathological phenomenon, introduced by Helmut Sies in 1985, as a disbalance between the levels of pro-oxidants and antioxidants with an abundance of the pro-oxidants, leading to disturbance/disruption in the redox signaling and consequent molecular damage[1,2]. It has been found to be one of the major contributing factors in diverse diseases such as malaria, gastropathy, cancer, diabetes, neuropathies, myopathies, cardiovascular disease, inflammation, and hepatitis [3-8]. In order to counteract the oxidative stress, antioxidants are called upon for action. An antioxidant can be defined as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [9]. A variety of antioxidants are present within the cells as a part of the endogenous defense arsenal to prevent the deleterious effects of potentially harmful reactive oxidants that are spontaneously generated within the system under physiological conditions or pathologically generated at significantly

deleterious amounts during diseases/cellular damage [10]. Reactive oxidants like hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and superoxide radical ($O_2^{\bullet-}$) are generated during normal aerobic cellular metabolism in the coral host and by photosynthesis in the endosymbiotic algae present within the coral tissue [11]. Even in the higher organisms including human, a basal amount of reactive oxidants are continuously produced within the cells the primary source of which being the inhaled oxygen. Around 1-2% of consumed oxygen (per day) goes into spontaneous ROS generation [12]. In this regard, mitochondrial electron transport chain is the predominant source of intracellular reactive oxygen species (ROS) generation (accounting to around 90%) wherein electron leakage occurs while transfer through the respiratory chain multiprotein complexes in the process of ATP production [12]. The leaked electrons lead to partial reduction of molecular oxygen thereby producing $O_2^{\bullet-}$ the progenitor ROS molecule. $O_2^{\bullet-}$ is subsequently dismutated to H_2O_2 by superoxide dismutase. H_2O_2 is subsequently either neutralized/detoxified to water or may be converted to OH (the most damaging ROS) by other proteins in presence of divalent metal ions including Fe^{2+}[13]. Reactive oxidants are also produced in other organelles like peroxisomes and endoplasmic reticulum during instances of cellular metabolism, protein folding, unsaturated fatty acid production, phagocytic clearance of invading pathogenic entities, immunological processes including inflammatory reactions as well as during xenobiotic metabolism [14]. Apart from ROS, reactive nitrogen species (RNS) like free radical form of nitric oxide ($^{\bullet}NO$), and peroxynitrite ($ONOO^{-}$) are also physiologically toxic reactive oxidants (at high concentrations) which are produced in the system under different physiological conditions including cellular metabolism as well as during pathological states of the cell [14]. Both ROS and RNS have physiological roles in cell signaling; however, overaccumulation beyond physiological limits prove detrimental to the cells whereupon cellular macromolecules including DNA, proteins and lipids are severely damaged and rendered functionally inactive [13]. In order to neutralize the oxidative stress, endogenous antioxidant defense arsenals of the cells including proteins like superoxide dismutase, peroxidase, catalase, peroxiredoxin and thioredoxin systems as well as peptide antioxidant like glutathione persistently operate to restore physiological redox homeostasis [15]. Often, sustained oxidative stress, during diverse cellular pathologies, overpowers the endogenous antioxidant levels thereupon demanding antioxidant supplementation to cope up the cellular damage during diseases. Therefore, synthetic and natural antioxidant compounds are always in huge demand and these compounds can thus be effective against excessive production of reactive oxidants and hence management of cellular pathologies and diseases.

As mentioned above, antioxidants are highly effective against different diseases where reactive oxidants and oxidative stress play an important role **(Figure. 1**).

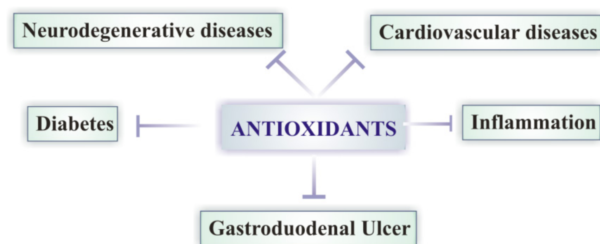


Figure 1. Effect of antioxidants against different pathologies (⊥ indicates inhibition).

Schiff bases (imines) are well known for their wide applications and are useful intermediates in organic synthesis [16]. These compounds have intrinsic biological activities including anti-inflammatory, analgesic, antimicrobial, anticonvulsant, antitubercular, anticancer, antioxidant, anti-helminthic [17,18]. On the

other hand, molecules having amide linkage show various biological activities [19]. The amide group is widely present in the drugs, intermediates, pharmaceuticals, and natural products. It is also available in large number of industrial products including polymers, detergents and lubricants [20,21]. Therefore, in this work some novel imines have been synthesized by condensation reaction of substituted benzaldehyde with substituted anilines and subsequently their antioxidant activity was evaluated.

Materials method and results

Synthesis of imines

The imines were synthesized via conjugation of differentially substituted aldehydes and different substituted amines (**Scheme 1**) [22] .

Synthesis of N-(4-methoxyphenyl)-1-(3-nitrophenyl)methanimine (I-1)

The reaction mixture of p-Anisidine (97.69 mg, 1.2 equiv. 0.7932m mol), m-nitrobenzaldehyde (100 mg, 1 equiv. 0.766 mmol) and Na_2SO_4 (93.88 mg, 1 equiv. 0.661 mmol) in dichloromethane (DCM) (2 mL) was stirred for overnight at room temperature. After the reaction, this mixture was checked by thin layer chromatography (TLC) using the developing solvent (25% ethyl acetate in hexane; R_f value 0.54). The colour of the crude mixture was slightly brown. The crude mixture was subsequently purified by column chromatography using 10% ethyl acetate in hexane. The solvent was evaporated & the purified product was collected. The compound was isolated as a blackish solid (**Scheme 1**).

Synthesis of N-(4-fluorophenyl)-1-(p-tolyl) methanimine (I-2)

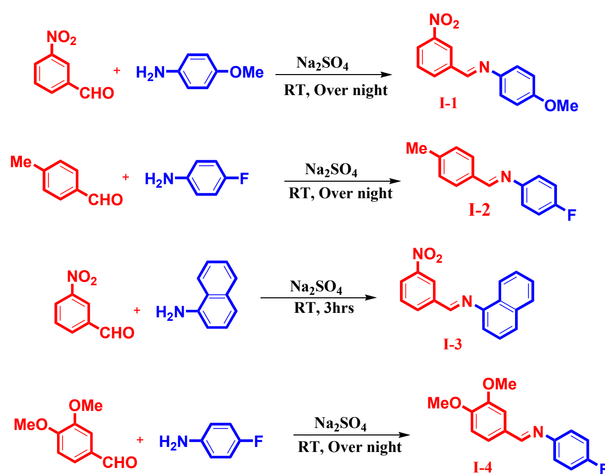
The reaction mixture 4-fluroaniline (110.89 mg, 1.2 equiv. 0.998 mmol), 4-methylbenzaldehyde (100 mg, 1 equiv. 0.83 mmol) and Na_2SO_4 (118.20 mg, 1 equiv. 0.83 mmol) in DCM (2 mL) was stirred for overnight at room temperature. After the reaction, this mixture was checked by TLC using the developing solvent (10% ethyl acetate in hexane; R_f value 0.35). The colour of the crude mixture was slightly brown. The crude mixture was subsequently purified by column chromatography using 5% ethyl acetate in hexane. The compound was isolated as yellowish liquid (**Scheme 1**).

Synthesis of (E)-N-(naphthalen-1-yl)-1-(3-nitrophenyl)methanimine (I-3)

The reaction mixture 1-napthyl amine (132.45 mg, 1.4 equiv. 0.93 mmol), 3-nitrobenzaldehyde (100 mg, 1 equiv. 0.66 mmol) and Na_2SO_4 (93.88 mg, 1 equiv. 0.66 mmol) in DCM (2 mL) was stirred for 3 hours at room temperature. After the reaction, this mixture was checked by TLC using the developing solvent (20% ethyl acetate in hexane; Rf value 0.69). The colour of the crude mixture was reddish black. The crude mixture was chromatographed over a silica gel column by running through 5% ethyl acetate in hexane. The crude mixture was subsequently purified by column chromatography using 5% ethyl acetate in hexane. The compound was isolated as reddish black solid (**Scheme 1**).

Synthesis of 1-(3,4-dimethoxyphenyl)-N-(4-fluorophenyl)methanimine (I-4)

The reaction mixture of 3,4-dimethoxybenzaldehyde (100 mg, 1 equiv. 0.6 mmol), 4-fluro aniline (93.6 mg, 1.4 equiv. 0.84 mmol) and Na_2SO_4 (85.46 mg, 1 equiv. 0.60 mmol) in DCM (2 mL) was stirred for overnight at room temperature. After the reaction, this mixture was checked by TLC using the developing solvent (30% ethyl acetate in hexane; R_f value 0.57). The colour of the crude mixture was slightly blackish red. The crude mixture was subsequently purified by column chromatography using 15% ethyl acetate in hexane. The solvent was evaporated & the purified product was collected. The compound was isolated as yellowish solid (**Scheme 1**).



Scheme 1: Synthetic scheme of imines derivatives. RT: room temperature**.**

Synthesis of Amides

All the amide derivatives have been synthesized using standard N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) coupling method (**Scheme 2**) [23]

Synthesis of N-(4-fluorophenyl)cinnamamide (A-1)

To a solution of cinnamic acid (100 mg, 1 equiv. 0.67 mmol), 4-fluroaniline (74.99 mg, 1equiv. 0.68 mmol) and 4-Dimethylaminopyridine (DMAP) (catalytic amount) in DCM, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) (157.12 mg, 1.5 equiv. 1.012 mmol) was added at 0ºC. Then the reaction mixture was stirred at room temperature for overnight until completion of reaction. The product was checked by TLC using the developing solvent (20% ethyl acetate in hexane; R_f value 0.58). The colour of the crude mixture was brownish yellow. The crude mixture was subsequently purified by column chromatography using 15% ethyl acetate in hexane. The solvent was evaporated & the purified product was collected. The compound was isolated as yellowish solid (**Scheme 2**).

Synthesis of N-(3-methoxyphenyl) cinnamamide (A-2)

To a solution of cinnamic acid (100 mg, 1 equiv. 0.67 mmol), m-Anisidine (83.12 mg, 1 equiv. 0.67 mmol) and DMAP (catalytic amount) in DCM, EDC hydrochloride (157.113 mg, 1.5 equiv. 1.012 mmol) was added at 0ºC. Then the reaction mixture was stirred at room temperature for overnight until completion of reaction. The product was checked by TLC using the developing solvent (30% ethyl acetate in hexane; R_f value 0.48). The colour of the crude mixture was reddish brown. The crude mixture was subsequently purified by column chromatography using 10% ethyl acetate in hexane. The solvent was evaporated & the purified product was collected. The compound was isolated as orange solid (**Scheme 2**).

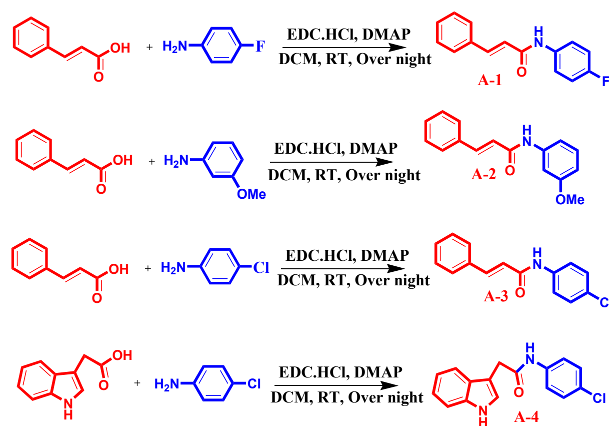
Synthesis of N-(4-chlorophenyl)cinnamamide (A-3)

To a solution of cinnamic acid (100 mg, 1 equiv. i.c 0.67 mmol), 4-chloaniline (85.71 mg, 1 equiv. 0.67 mmol) and DMAP (catalytic amount) in DCM, EDC hydrochloride (157.1 mg, 1.5 equiv. 1.01 mmol) was added at 0ºC. Then the reaction mixture was stirred at room temperature for overnight until completion of reaction. The product was monitored by TLC using the developing solvent (20% ethyl acetate in hexane; R_f value 0.56). The colour of the crude mixture was brownish yellow. The crude mixture was subsequently purified through column chromatography using 10% ethyl acetate in hexane. The solvent was evaporated & the purified product was collected. The compound was isolated as white solid (**Scheme 2**).

Synthesis of N-(4-chlorophenyl)-2-(1H-indol-3-yl)acetamide (A-4)

To a solution of indole-3-acetic acid (100 mg, 1 equiv. 0.57 mmol), 4-chloaniline (72.39 mg, 1 equiv. 0.570 mmol) and DMAP (catalytic amount) in DCM, EDC hydrochloride (132.73 mg, 1.5 equiv. 0.85 mmol) was added at 0ºC.

Then the reaction mixture was stirred at room temperature for overnight until completion of reaction. The product was monitored by TLC using the developing solvent (20% ethyl acetate in hexane; R_f value 0.41). The colour of the crude mixture was reddish brown. The crude mixture was subsequently purified through column chromatography using 10% ethyl acetate in hexane. The solvent was evaporated & the purified product was collected. The compound was isolated as yellowish solid (**Scheme 2**).



Scheme 2: Synthetic scheme of Amide derivatives. RT: room temperature**.**

Free radical scavenging activity in vitro [2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Since antioxidants can scavenge free radicals, therefore free radical scavenging ability of these synthesized molecules have been evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. DPPH is a stable free radical that can accept hydrogen radical or an electron and become a stable diamagnetic molecule. The antioxidants can scavenge the DPPH by donating hydrogen as visualized by discoloration of the DPPH radical from purple to yellow [24]. The assay system contained 1 mL of imine's and amide's solution at varying concentrations of 10-100 µM and 4 mL of DPPH (0.15 mM) in methanol (80 % in water v/v). The reaction mixture was mixed well by trituration. It was allowed to stand for 30 min at room temperature away from light. Ascorbic acid at the same concentration was taken as positive control. The absorbance of the solution was measured spectrophotometrically at 517 nm and from the decrease of absorption, the antioxidant activities of synthesized imines were determined.

Results indicated that, all the synthesized compounds showed significant DPPH free radical- scavenging activity (**Table-1**). Among the synthesized molecules **I-1** (imine) and **A-1** (amide) showed greater activity; therefore, concentration-dependent activity assays were performed for **I-1** and **A-1**. Results indicated that **I-1** and **A-1** exhibited 80% and 79% DPPH scavenging activity at a concentration of 100 µM respectively. (**Figure-2**)

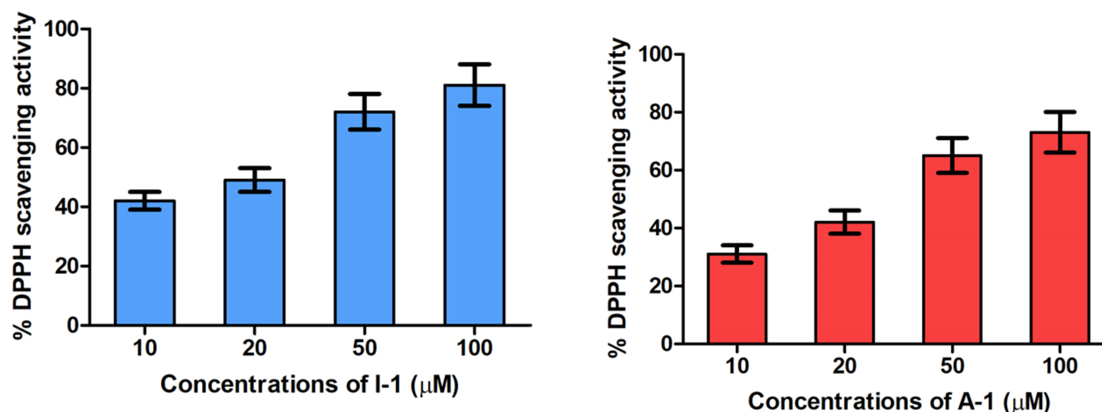


Figure 2. DPPH free radical-scavenging activity of imines I-1 and A-1 at various concentrations

Determination of *in vitro* antioxidant property by ferric-reducing antioxidant power (FRAP) assay

The reducing activity of antioxidants was evaluated by ferric-reducing antioxidant power (FRAP) assay as described previously [24]. The FRAP assay was performed in a 96-well microplate. FRAP reagent was prepared by mixing 10 mL acetate buffer (200 mM, pH 3.6), 1 mL of 2,4,6-tripyridyl-S-triazine (TPTZ) solution (10 mM in 40 mM HCl), and 1 mL of ferric chloride solution (20 mM) in distilled water. The mixture was kept in a water bath at 37°C for 1 h. The imines and amides under investigation were dissolved in methanol at various concentrations in the range of 10–100 μM. Freshly prepared FRAP solution (175 μL) was placed in each well and 25 μL of the imines and amides solution was added to it. All tests were done in triplicate and repeated several times to minimize error. A microplate reader monitored the absorbance at 595 nm at various time intervals for 80 min. The absorbance of a mixture of 175 μL FRAP solution and 25 μL methanol, considered as blank, was monitored in parallel and subtracted from that of the sample at each time interval to calculate the absorbance change (ΔA).

The FRAP value at time interval t (FRAP $value_t$) was calculated according to the formula [25]

$$\textbf{FRAP value}_t\ \textbf{(M)} = (\Delta a_t Fl / \Delta a_t Fe^{2+})\ X\ 10^{-5}$$

where $\Delta a_t Fl$ is the absorbance change after the time interval t (65 min) relative to the tested amides and imines at the concentration of 100 μM and $\Delta a_t Fe^{2+}$ is the absorbance change of ferrous sulphate at the same concentration. The assay was also performed with some known antioxidant (positive control) such as ascorbic acid at the same concentration.

The FRAP assay is based on the measurement of the ability of a substance to reduce Fe (III) to Fe (II); greater the reducing ability greater the antioxidant property. Antioxidants reduce the colorless Fe^{III}-TPTZ to a blue colored Fe^{II}-TPTZ complex, which results in an increase in the absorbance at 595 nm giving a FRAP value. Higher FRAP value indicates greater reducing (i.e. antioxidant property) ability of the compound. Absorbance changes at 595 nm were measured at different time intervals **(Figure. 2)** for different concentrations of imines and amides (**Figure. 2**) after a fixed time of 6 min. FRAP values at 6 min and 65 min were calculated (**Table. 1**) from the equation as described above. Results clearly indicated that imines and amides show considerable reducing ability [Fe(III) to Fe(II)]. Among the synthesized molecules **I-1** (imine) and **A-1** (amide) showed greater activity, therefore concentration-dependent activity assays were

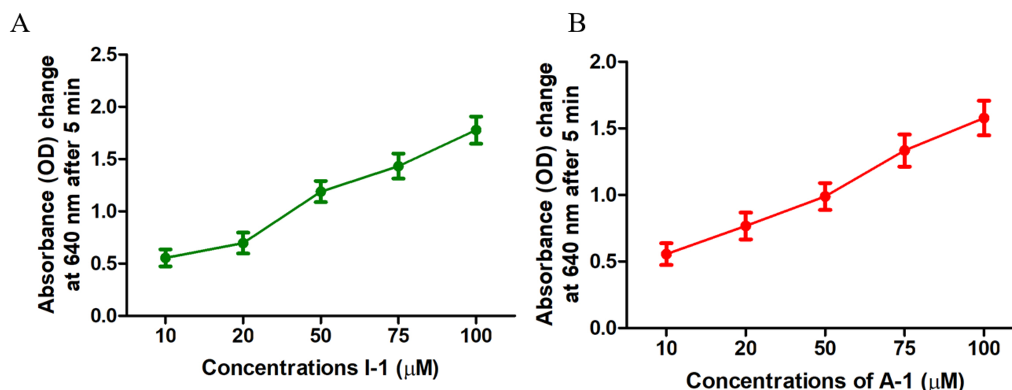


Figure 3: Concentration dependent ferric reducing antioxidant property of activity of I-1 and A-1

performed for **I-1** and **A-1** (**Figure-3**).

Table 1: Absorbance change for 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and ferric-reducing antioxidant power (FRAP) values of different test compounds at and 65 min.

Compounds	DPPH Change of absorbance (ΔA_{517})	FRAP Value (µM) Mean ± SD
I-1	**0.82 ± 0.02**	**36.52 ± 2.6**
I-2	0.72 ± 0.015	21.32 ± 2.1
I-3	0.61 ± 0.016	18.54 ± 2.7
I-4	0.59 ± 0.082	12.52 ± 2.1
A1	**0.78± 0.025**	**29.47 ± 2.6**
A-2	0.63 ± 0.021	12.24 ± 1.9
A-3	0.54 ± 0.019	17.15 ± 2.3
A-4	0.42 ± 0.071	14.91 ± 1.7

Conclusion & future impact

In the present study, the synthesized imine (**I-1**) and amide (**A-1**) showed considerable antioxidant activities as observed from DPPH free radical scavenging and FRAP-based antioxidant activity assays in the *in vitro* system. The preliminary results are highly promising and the compounds can be used for subsequent cell-based assays for checking their biological potency in the *in vitro* followed by *in vivo* experimental models. Since these small molecules can prevent reactive pro-oxidant mediated oxidative stress in the *in vitro* assay systems, it may be expected that these compounds would also exhibit anti-oxidant actions in the complex biological systems and therefore be effective against a wide spectrum of human diseases such as cancer, diabetes, neuropathies, myopathies, cardiovascular disease, gastric ulcer and hepatitis where mitochondrial pathologies play an important role [3,26]. The outcome from the present study therefore highly warrant further explorative studies with these compounds for determining their effective anti-oxidant concentrations in the biological systems (*in vitro* and *in vivo*) as well as toxicity evaluation in the cell lines and subsequently animal models to check for potential toxic/lethal effects (if at all) before progressing for in depth mechanistic and sub-cellular target identification studies. The present study holds clinical relevance as these novel scaffolds can be used by the synthetic chemists and pharmaceutical researchers for new-generation anti-oxidant drug development after precise toxicological and regulatory studies.

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