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In- vitro antioxidant and antibacterial activities of the four synthesized indole derivatives

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ABSTRACT

The indole nucleus seems to be a promising basis for design and synthesis of new derivatives able to protect oxidative stress in a variety of acute and chronic pathologies. The paper presents an overview of indole derived compounds in which inhibitory action has been demonstrated against potent microbes and also tested for antioxidant activity. Cellular damage caused by reactive oxygen species has been implicated in several diseases and hence antioxidants have significant importance in human health. *In vitro* evaluation of antioxidant and antibacterial potentials of the four compounds viz. I_1, I_2, I_3 and I_4 showed significant results. Compound I_4 showed promising results for reducing power, hydroxyl free radical and superoxide scavenging assays when compared with standard drug. The same showed highest inhibitory activity against Gram positive organism, where as I_1 showed least effect and I_2, I_3 were moderate in both the activities. Eventhough all the four compounds are indole derivatives, their change in the potencies may be due to the structural differences.
Keywords: antibacterial, hydroxyl radical, indole derivatives, reducing power, superoxide scavenging.

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INTRODUCTION

Owing to the great structural diversity of biologically active indoles, it is not surprising that the indole ring system has become an important structural component in many pharmaceutical agents [1]. Substituted indoles have been referred to as privileged structures since they are capable of binding to many receptors with high affinity [2]. The indole skeleton is one of the most attractive frameworks with a wide range of biological and pharmacological activities. This physiologically important nucleus is abundantly found in therapeutic agents [3] as well as in natural products. The occurrence and availability of indoles in nature is widespread and a large number of them exhibit biological activity [4] and some of the indole derivatives posses cytotoxic activity [5][6][7]. Replacement of indole ring by other heterocycles is often accompanied by loss of the biological activity. The indole ring system is found in diverse naturally occurring molecules including Tryptophan -an essential amino acid [8]; 3-indoleacetic acid the main plant growth hormone of higher plants; and serotonin a bioregulator, which is one of the key neurotransmitters in animals that plays an essential role in our mental health [9]. Skatole -3-methylindole [10], arising from the digestion of proteins, is responsible for the repulsive odour of faeces. chelonin A [11] isolated from a marine sponge of the *Chenolaplysilla* species showed potent antimicrobial and anti-inflammatory activities. Discodermindol which was discovered from *Discodermia polydiscus* showed cytotoxic activity [12][13],and they also possess anti-inflammatory properties [14].

Of the compounds interfering with the effects of stress, indole derivatives create a distinct chemical and pharmacological group. Some of them seem to exhibit a promising battery of useful properties. By the interaction of the radical with other protein radicals, cross-links might be generated in proteins, thus leading to their impairment. An antioxidant is a molecule capable of showing or preventing the oxidation of other molecules. Oxidation process is one of the most important routes for producing free radicals in food, drugs and even living system, and also participates in numerous pathological processes. A balance between tissue concentration of ROS and natural antioxidative mechanisms in tissue is disturbed under such conditions. This may either result from an increased local production of ROS or from exhaustion of the antioxidant capacity of the tissue. In cellular oxidation reactions, super oxide radical normally is formed first, and its effect can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. The damaging action of the hydroxyl radical is the strongest among free radicals [15]. Reactive oxygen species produced by ultraviolet light, ionizing radiation, chemical reactions and metabolic process have numerous pathological effects, such as causing lipid peroxidation, protein peroxidation, DNA damage and cellular degeneration related to cardiovascular disease, ageing, cancer, inflammatory diseases, and a variety of other disorders [16][17][18]. Low level of antioxidants or inhibition of antioxidant enzymes causes oxidative stress and may damage or kill cells [19]. It has been suggested that antioxidant may amend cellular oxidative status and prevent biologically significant molecules such as DNA, proteins, and membrane lipids from oxidative damage and as a result lesser the risk of several chronic diseases including cancer and cardiovascular diseases [20].

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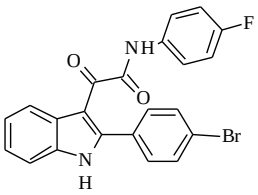
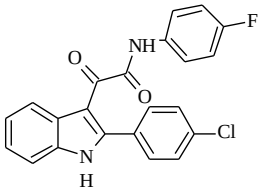
Infectious diseases are the world's major threat to human health and life, accounting for almost 50,000 deaths per day [21]. The discovery of antibiotics and their uses as chemotherapeutic agents kindled a brief in the medical fraternity that antibiotics will lead to the eradication of all the infectious diseases. The development of resistant microorganism on prolonged exposure to existing antimicrobial agents has been known for a long time [22]. There is arising prevalence of pathogenic microorganisms which is resistant to the newer or modern antibiotics that have been introduced in the last three decades [23]. Thus diseases and the causative agents, which were once thought to be controlled by antibiotics, are returning in new forms resistant to antibiotics [24]. Thus the development of new sources of antibiotics is a global challenge for preoccupying research institutions, pharmaceutical companies and academia [25].

Hence, the present work reports the *in vitro* antioxidant and antibacterial properties of the synthesized indole derivatives. The reducing power, hydroxyl free radical and superoxide scavenging activity were assayed using standard methods. The inhibiting property of the compounds on certain bacteria which causes diseases was also studied. Results of such studies are reported in this paper.

MATERIALS AND METHODS

Compounds and materials

Table 1: Details of compounds selected for the activities.

Compound No.	Structure	Molecular Formula and Name	Molecular weight
I_1		$C_{22}H_{14}BrFN_2O_2$ 2-[2-(4-bromophenyl)-1*H*-indol-3-yl]-N-(4-fluorophenyl)-2-oxoacetamide.	437.26
I_2		$C_{22}H_{14}ClFN_2O_2$ 2-[2-(4-chlorophenyl)-1*H*-indol-3-yl]-N-(4-fluorophenyl)-2-oxoacetamide.	392.81

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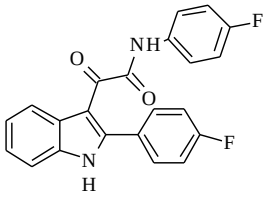
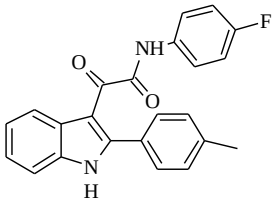
I_3		**$C_{22}H_{14}F_2N_2O_2$** N-(4-fluorophenyl)-2-[2-(4-fluorophenyl)-1*H*-indol-3-yl]-2-oxoacetamide.	376.35
I_4		**$C_{23}H_{17}FN_2O_2$** N-(4-fluorophenyl)-2-[2-(4-methylphenyl)-1*H*-indol-3-yl]-2-oxoacetamide.	372.39

Four synthesized indole derivatives with possible biological activities were procured from the Department of Chemistry, Kuvempu University, Karnataka, India. The details of the compounds are shown in table 1. All solvents and other chemicals used were of analytical grade and obtained from Merck, Mumbai, India.

Antioxidant activity

i. Total reduction ability by Fe^{3+}- Fe^{2+} transformation

The reducing power of compounds was determined according to the method of Oyaizu [26]. Different concentrations of compounds (50, 100, 200 and 300 µg) in 1 ml of methanol were mixed with phosphate buffer (2.5 ml, 2.0 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 mins, then cooled rapidly and mixed with trichloroacetic acid (2.5 ml, 10%), which was then centrifuged at 3000 rpm for 10 mins. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%), and the amount of iron (II)-ferricyanide complex was determined by measuring the absorbance at 700 nm. Increased absorbance of the reaction mixture indicates increased reduction capability, Fe (III) reduction is often used as an indicator of electron-donating activity. BHT (Butylated hydroxy toluene) was used as a standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was taken and is expressed as mean ± standard deviation.

ii. Hydroxyl radical scavenging assay

The assay was performed as described by Halliwell *et al*. method [27] and BHT was used as a standard. All solutions were prepared freshly. 1 ml of the reaction mixture contained 100 µl of 2-deoxyribose (28 mM in phosphate buffer, pH 7.4), 500 µl solution of various concentrations of the compounds (50, 100, 200 and 300 µg), 200 µl of $FeCl_3$ (200 µM) and EDTA

(1.04 mM), 100 µl of H_2O_2 (1.0 mM) and ascorbic acid (1.0 mM). After an incubation period of 1 h at 37 °C, 1 ml thiobarbituric acid (1%) and 1 ml of trichloroacetic acid (2.8%) were added to the test tubes and were incubated in boiling water bath for 20 mins, the extent of deoxyribose degradation was measured by the TBARS reaction. After cooling; absorbance was measured at 532 nm against the blank solution containing deoxyribose and buffer. The percentage of H_2O_2 scavenging was calculated as:

$$\% \text{ Scavenged } [H_2O_2] = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where, A_{blank} is the absorbance of the blank in absence of sample, and A_{sample} is the absorbance in the presence of sample.

iii. Superoxide scavenging activity

Measurement of superoxide anion scavenging activity was performed with NBT/NADH/PMS system based on the Nishimiki method [28]. About 1 ml of NBT (Nitroblue tetrazolium) solution (156 µM in 100 mM phosphate buffer, pH 7.4), 1ml NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution (100, 200, 300 and 400 µg) in methanol were mixed. The reaction started by adding 100 µl of phenazine methosulphate solution (60 µM in 100 mM phosphate buffer, pH 7.4) to the mixture and BHT was used as a standard. The reaction mixture was incubated at 25^0C for 5 mins, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage of super oxide anion scavenging was calculated as:

$$\% \text{ Scavenged of superoxide anion} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where, A_{blank} is the absorbance of the blank in absence of sample, and A_{sample} is the absorbance in the presence of the sample.

Bacterial susceptibility testing:

In vitro antibacterial activity of the crude extracts was studied against Gram-negative and Gram-positive bacteria by the agar well diffusion method [29]. Nutrient agar (Hi Media, India) was used as the bacteriological medium. 10 mg of test compounds were dissolved in DMSO (1ml), thus giving a final concentration of 1mg/0.1ml. Pure DMSO was taken as the negative control and 10 mg/ml Ciprofloxacin as the positive control. The bacterial strains used for screening of antibacterial activity were collected from different infectious status of the patients who had not taken any antibacterial drugs for at least 2 weeks with the help of authorized physician, in the district health centre of Shivamogga, Karnataka, India. The clinical isolates were identified following the standard method [30]. One day prior to the test, the microorganisms were inoculated into sterilized nutrient broth tubes and incubated at 37^oC for 24 hrs. The organisms *Staphylococcus aureus* (gram positive) and *Pseudomonas aeruginosa*

(gram negative) were sub-cultured into sterile nutrient broth. After incubating the same for 24 hours, the growth thus obtained was used as inoculums for the test. 100 µl of each compound concentration and control compound were dropped into each, appropriate labeled well. The inoculated plates were kept in the refrigerator for 1 h to allow the extracts to diffuse into the agar. The Nutrient agar plates were incubated at 37^{o}C for 24 hrs and experiment was conducted in triplicates. Subsequently, the plates were examined for microbial growth inhibition and the inhibition zone diameter was measured to the nearest mm.

Statistical analysis:

The values were expressed as mean ± SEM. Statistical analysis of data was performed using ANOVA followed by student t-test to study the differences amongst the means [31]. Values of $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

In vitro antioxidant activity

Table 2: Total reduction ability of the 4 compounds by Fe^{3+}- Fe^{2+} transformation.

Compounds	50 (µg) %Inhibition	100 (µg) %Inhibition	200 (µg) %Inhibition	300 (µg) %Inhibition
I_1	2.00 ± 0.04**	7.64 ± 0.21**	12.47 ± 0.11**	28.18 ± 0.40**
I_2	7.26 ± 0.21**	31.74 ± 0.11**	66.46 ± 0.20**	78.12 ± 0.44**
I_3	4.04 ± 0.12**	14.70 ± 0.03**	35.27 ± 0.10**	57.56 ± 0.42**
I_4	19.80 ± .17**	33.60 ± 0.27**	75.30 ± 0.13**	142.06 ± 0.78**
BHT	115.46 ± 4.70	174.20 ± 3.92	362.36 ± 2.10	605.24 ± 0.52

The values are the mean of duplicates ± S.E. ** P<0.01 compared to standard.

Table 3: Hydroxyl radical scavenging assay of the 4 compounds.

Compounds	50 (µg) %Inhibition	100 (µg) %Inhibition	200 (µg) %Inhibition	300 (µg) %Inhibition
I_1	4.89 ± 0.03**	6.57 ± 0.30**	12.82 ± 0.09**	14.77 ± 0.26**
I_2	8.32 ± 0.16**	14.50 ± 0.33**	17.55 ± 0.13**	26.38 ± 0.36**
I_3	6.38 ± 0.35**	12.50 ± 0.33**	21.48 ± 0.37**	25.69 ± 0.27**
I_4	10.46 ± 0.17**	22.66 ± 0.21**	36.45 ± 0.37**	66.18 ± 0.40**
BHT	17.63 ± 0.35	32.47 ± 0.26	48.44 ± 0.42	75.88 ± 0.39

The values are the mean of duplicates ± S.E. ** P<0.01 compared to standard.

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Table 4: Superoxide scavenging activity of the 4 compounds.

Compounds	100 (µg) %Inhibition	200 (µg) %Inhibition	300 (µg) %Inhibition	400 (µg) %Inhibition
I_1	9.64 ± 0.18**	11.68 ± 0.25**	17.60 ± 0.09**	22.81 ± 0.60**
I_2	12.51 ± 0.34**	22.48 ± 0.46**	30.62 ± 0.35**	32.11 ± 0.23**
I_3	10.62 ± 0.24**	32.53 ± 0.05*	37.58 ± 0.33**	39.12 ± 0.23**
I_4	12.50 ± 0.33**	47.59 ± 0.36**	48.45 ± 0.37**	80.85 ± 0.40**
BHT	20.64 ± 0.21	54.08 ± 1.59	57.82 ± 0.17	100.50 ± 0.23

The values are the mean of duplicates ± S.E. ** P<0.01 compared to standard.

Indole derivatives have a heterocyclic aromatic ring structure with high resonance stability and several different substituents on the ring, and this led the researchers to suspect antioxidant activity in these compounds on theoretical founds. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 710 nm. Increasing absorbance at 700 nm indicates an increase in the reductive ability. Table 2 shows the reducing power of the 2-phenyl indole derivatives at various concentrations. Compounds I_2 and I_4 showed highest reductive ability compared to compounds I_1 and I_3 which were active considerably. All compounds showed comparatively lesser but significant reductive ability than the standard BHT at all the concentrations. Table 3 shows the hydroxyl scavenging effect of the indole derivatives at various concentrations, which were generated by reaction of Ferric-EDTA together with H_2O_2 and ascorbic acid. The compound I_4 was noticed to prevent the action of hydroxyl radical against deoxyribose. Further compounds I_2 and I_3 were moderately effective to inhibit the degradation of deoxyribose by hydroxyl radical, where as compound I_1 recorded lowest inhibition. It is well known that hydrogen peroxide generated by human polymorph nuclear leukocyte alter human IgG to a fluorescence product which is antigenic in reacts with IgM and IgA rheumatoid factors [32]. The formation of the alter IgG is inhibited by catalyses, which also inhibit the binding of this antigenic IgG to rheumatoid factors. Thus the inhibition of hydrogen peroxide formation may be effective in the treatment of inflammatory disorders. Zeynep *et al*. [33] has also showed that indole derivatives are a potent hydroxyl radical scavenger. The superoxide anion derived from dissolved oxygen by phenazine methosulphate/NADH coupling reaction reduces NBT. The decrease in the absorbance at 560 nm with the compounds thus indicates the consumption of superoxide anion in the reaction mixture. The results of the inhibitory effects of 2-phenyl indole derivatives at various concentrations are presented in table 4. Additionally compounds I_2 and I_3 showed appreciable scavenging of superoxide anion compared to compound I_1. Whereas compound I_4 showed very high activity compared to that of the standard BHT at all the concentrations. The occurrence of carbon and nitrogen centered radicals may explain the possible antioxidant mechanism of indole derivatives [34] that was proved by electroanalytical studies [35]. Therefore, the observation of different effects of synthetic compounds on various antioxidant activities was not surprising since the mechanism of production of oxidative stress by these methods were different [36][37][38].

ISSN: 0975-8585

Antibacterial activity

Table 5: Antibacterial activities of the 4 compounds.

Compounds	Zone of inhibition (mm)			
	S. aureus		*P. aerugenosa*	
	Sputum	Pus	Sputum	Urine
I_1	8.75±0.28**	16.10 ± 0.28**	12.14 ± 0.31**	11.22 ± 0.39**
I_2	12.72±0.23**	20.16 ± 0.37**	14.20 ± 0.34**	16.00 ± 0.13**
I_3	12.21±0.24**	16.02 ± 0.23**	15.19 ± 0.33**	14.96 ± 0.17**
I_4	19.86±0.19**	20.03 ± 0.32**	17.70 ± 0.38**	19.98 ± 0.16**
Ciprofloxacin	27.94±0.36	29.83 ± 0.12	28.74 ± 0.16	29.68 ± 0.32

The values are the mean of triplicates ± S.E. ** P<0.01 compared to standard.

Synthesized compounds were screened for their antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aerugenosa.* The maximum activity was found in compound I_4 for both the Gram strains, which was followed by I_2 and I_3. Where as I_1 showed very less significant results against both the strains (Table 5). The inhibition zones obtained by compound I_4 against both the organism samples isolated from sputum, pus and urine are significantly high and similar to that of the standard ciprofloxacin. The antibacterial activity was observed to be high towards *Staphylococcus aureus* than *Pseudomonas aerugenosa,* which correlates to the fact that the cell wall in Gram-positive bacteria has a single layer, whereas the Gram-negative cell wall is a multi-layered structure [39][40], acting as a barrier to many environmental substances, including antibiotics [41]. According to Sibel *et al*. [42] the changes in zone produced by the compounds are mainly related to particular properties that depend on their respective chemical structures.

CONCLUSION

The above findings suggest that the four compounds exert significant antioxidant activity *in vitro* and also possess inhibitory effect against different bacteria. The effects are more evident for compound I_1. However, the activity level of the compounds can be more accurately evaluated in terms of MIC values as the zone of inhibition might be influenced by solubility and diffusion rate. In addition, *in vivo* studies are necessary to determine the toxicity of the active constituents, their side effects, circulating levels, pharmacokinetic properties and different pharmacological activities. Compound I_1 with no substitution at the indolic nucleus has the simplest structure and could be considered as a promising agent for further evaluation of the mechanism of *in vivo* antioxidant activity and other properties.

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