Prospect of developing a novel potent Antioxidant from an Antidepressant Agent

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ABSTRACT

Objective: Worldwide scientific investigations strongly suggest the urgent need of antioxidants in preventing the oxidative stress disorders triggered by reactive oxygen species (ROS) and free radicals. Based on multiplicity of action in pharmacological compounds, this study has been directed to search for antioxidant capacity in the antidepressant drug doxepin, having structural similarity with the known antioxidant flavonoid, quercetin.

Methods: Standard spectrophotometric procedures like Total antioxidant capacity assay (TAC assay), Ferric ion and cupric ion reducing power assays (FRAP& CUPRAC assay), Ferrous ion chelating activity assay, hydrogen peroxide and nitric oxide radical scavenging assays were conducted and the results were statistically analysed.

Results: The outcomes showed the antioxidant capacity of the drug to increase stably in a dose-dependent manner for TAC, FRAP and CUPRAC assays. Also almost similar results were observed in case of the ferrous ion chelating activity assay. Though, doxepin revealed a strong nitric oxide scavenging activity in all its concentrations, however, it showed a positive hydrogen peroxide scavenging activity only at highest concentration.

Conclusion: Thus, in this way this study enhances the scope of developing novel antioxidants from already known pharmaceutical compounds that are in routine therapeutic usage.

Keywords: *Free radicals; Antioxidants; Doxepin, TAC assay; Nitric oxide scavenging activity*

INTRODUCTION

Several different processes of biochemical reactions are essential to sustain the life of a human being. These processes lead to formation of active bio-molecules some of which are free radicals and reactive oxygen species (ROS) [1]. Regular contacts with various environmental pollutants like pesticides, toxic chemicals, urban air pollutants, gasoline exhaust or indirect exposure to cigarette smoking, radiations and even physical stress are known to synthesize a number of reactive molecular species in our bodies [2]. Such active molecules are often responsible for degradation of proteins, lipids and DNA activation of procarcinogens, inhibition of cellular and antioxidant defense systems, alteration in calcium homeostasis, and gene expression, progression of abnormal proteins leading finally to significant changes in the pathological systems of human being [3 -5]. The agents that can inhibit action of these free radicals and ROS are termed as antioxidants. Certain substances like vitamins C and E, beta-carotene, and proanthocyanidine, minerals like zinc and selenium, enzymes such as glutathione, superoxide dismutase and catalase have been studied extensively for their role as potent antioxidants against tumorogenesis and

carcinogenesis [5 -8]. According to Halliwell [6] an antioxidant is a substance that is capable of delaying, preventing or eliminating the damaging capacity of a targeted molecule. Sies et al [9] have suggested that there may be a link between increased levels of ROS and distortion of enzymatic and nonenzymatic antioxidants in age related diseases. Antioxidants have been found to act through various modes. Oxidation reactions can be prevented by free radicals, inhibition of free lipid radical formation, conversion of hydroperoxides through reduction, quenching singlet oxygen, chelation of metals to form stable compounds and inhibition of pro-oxidative enzymes [10 – 14].

The polyphenolic compounds isolated from a variety of vascular plants are called flavonoids. These are present in plants as glycosylated derivatives and are responsible for the brilliant blue, orange and scarlet colours of leaves, flowers and fruits. Numerous seeds, nuts, grains, spices, medicinal plants as well as beverages like tea, red wine and even beer also carry moderate to reasonably high amounts of flavonoids [15]. Flavonoids have been studied extensively for various beneficial properties. They have been found to have anti-inflammatory, antihistaminic and vasodilating actions. Flavonoids obtained from different plant sources have also been found to possess potent antimicrobial activity [16-19]. However, the antioxidant capacities of flavonoids have also been evaluated in great details [20].

Among the flavonoids quercetin represents a major component. Quercetin is widely distributed in nature and has been studied intensively for its action on prevention of low density lipoproteins by scavenging free radicals and chelating transition metal ions [21].

During the past several years a number of researchers from different parts of the world have repeatedly shown presence of moderate to highly potent antimicrobial action in a number of pharmaceutical compounds that are prescribed for different types of ailments [22-25]. Such studies provided an understanding that multiplicity of function of drugs is supposedly a rule rather than exception. Continuing with this understanding structure of the drug doxepin was evaluated and was observed to possess a great structural similarity with a flavonoid, particularly quercetin. Thus based on this observation the antidepressant compound doxepin was selected to determine its antioxidant property. The present study describes such a property in a greater detail.

MATERIALS AND METHODS

Drug: The drug of our choice doxepin was acquired from Sigma-Aldrich, USA. It was in the form of water-soluble pure dry powder and was preserved at 4^0C.

Chemicals and Reagents: Analytical grade chemicals and reagents were used in all the assays performed in this study. These were purchased from different vendors such as LobaChemie (Mumbai, India), Sisco Research Laboratory (SRL, Mumbai,India), Merck (Bangalore, India) and from HiMedia (Mumbai, India).

Preparation of the sample: Using Milli-Q grade water, a working solution of 1 mg/mL of the drug powder was freshly prepared for every analysis.

Procedures followed for identification of *in vitro* antioxidant and free radical scavenging activity

Estimation of total antioxidant capacity: For the determination of total antioxidant capacity of doxepin the phosphomolybdenum assay was followed. Methodologies were performed according to Prieto*et al* [22] which was based on the principle of reduction of Mo (VI) to Mo (V) by an agent and subsequent formation of a green phosphate/Mo(V) complex at acidic pH.The different concentrations of doxepin used in this experiment were 25, 50, 100, 200, 500 µg/ml. Absorbances were measured at 695 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank. A standard curve was plotted with different concentrations of ascorbic acid (1000, 500, 250, 125, 62.5 and 31.25 µg/mL). Ascorbic acid equivalent activity (AAEA) or the total antioxidant activity is expressed as the µg/mL equivalents of ascorbic acid. The following formula used for its calculation:

% of inhibition = $(A_o – A_1)/ A_o \times 100$, where A_o is the absorbance of the control (blank, without drug) and A_1 is the absorbance of the different drug solutions.

Estimation of ferric ion reducing capacity: This experiment was also performed following the methodologies as described by Prieto*et al* [26]. Concentrations of doxepin used were same as used in the previous one. The basic principle of this assay is that potent antioxidants reduce Fe3+ ions to Fe2+ which forms a Prussian blue coloured complex with ferrocyanide. The colour intensity in each reaction tube was measured at 700 nm in a UV-VIS spectrophotometer (Evolution 201, ThermoFisher) against the blank. Different concentrations of ascorbic acid (1000, 500, 250, 125,62.5 and 31.25 µg/mL) were used to prepare the standard curve.

Estimation of cupric ion reducing capacity: For determining the cupric ion reducing capacity of doxepin, the methods as elaborated by Apak*et al* [27] were followed. This experiment is based on the principle of formation of stable coloured complex between copper (I) and neocuproine, where the latter is used as the oxidizing agent for the reaction. Absorbancesof the reaction tubes containing different concentrations of doxepin (25, 50, 100, 200, 500 µg/ml) were taken at 450 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank. The standard curve was plotted with different concentrations of ascorbic acid (1000, 500, 250, 125, 62.5 and 31.25 µg/mL) and the results are expressed as µg/mL equivalents of the standard.

Estimation of ferrous ion chelating activity: This assay was conducted following the standard method as described byMukhopadhyay*et al* [28]. The assay reaction is initiated by ferrozineby combining with divalent iron to form a stable magenta complex species, the absorbance of which is measured at 562 nm. However the potent metal ion chelators presentcompetes with ferrozine in chelating the ferrous ions and thus the ferrozine-Fe 2+ complex formation is interrupted. Ethylene diamino tetra acetate (EDTA) was used as the positive control for this assay. The following formulawas used for determining the chelating activity of the drug– Ferrous ion chelating ability in % = [1-(test sample absorbance/blank sample absorbance)] X 100%.

Estimation of Hydrogen peroxide scavenging activity: This assay was performed following the modified method as elucidated by Mukhopadhyay*et al* [29]. Accordingly, ascorbic acid was used as positive control for the assay and the absorbancesof the reaction tubes were measured at 510 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank. The % of hydrogen peroxide scavenging activity of the drug was calculated as given below:

% of scavenging activity = (A_{test} – A_{blank}) X 100, where $A_{blank}$and $A_{test}$are the absorbances of the blank solution and test solution respectively.

Estimation of nitric oxide scavenging activity: Nitric oxide is a very critical free radical having numerous toxic effects. It also has significant role in inflammatory conditions like juvenile diabetes, multiple sclerosis, arthiritis and ulcerative colitis [30]. To determine the nitric oxide scavenging activity of the antidepressant drug the procedure as described by Chaudhuri et al [31] has been followed. As per the methodology, nitric oxide generated from sodium nitroprusside interacts with oxygen present in the reaction system to form the highly reactive peroxynitrite anion (ONOO -) and this can be measured by GriessIllosvoy reaction [32]. Absorbances of the reaction tubes were measured at 546 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank. A standard curve with different concentrations of ascorbic acid as the positive control was also prepared. The percentage of nitric oxide radical scavenging activity of the drug solutions was calculated as given below:

% of scavenging activity = (Absorbance of control – Absorbance of test/ Absorbance of control) X 100

RESULTS

Estimation of total antioxidant capacity: The result of the experiment indicates that as there is an increase in the concentration of the antidepressant compound there is also an increase in its total antioxidant capacity. The bar diagram [Fig.1] which has been prepared depicting the obtained AAE values against the different concentrations of the drug, signifies that

doxepin has a moderately highmolybdenum ion reducing ability with the values ranging from 25.3 ± 1.47 AAE at 25 µg/mLand reaching its maximum to 174.35 ± 8.39 AAE at 500 µg/mL.

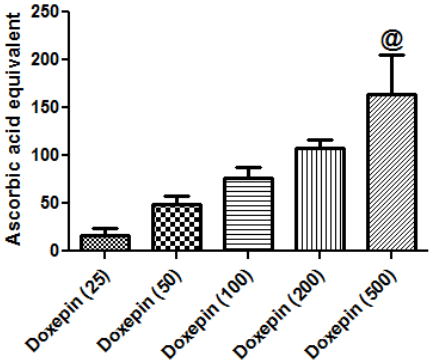

Fig.1 Bar graph showing the total antioxidant capacity of doxepin where the X-axis denotes the different concentrations of thedrug (µg/ml)

* @ means p<0.01, compared to Doxepin (25) and Doxepin (50).The assay was repeated three times (n=3). Values are expressed as mean ± standard deviation (SD).

Estimation of ferric ion reducing capacity: Through various studies worldwide it has been concluded that compounds having significant reducing power have great potency to act as electron donors and also can reduce the oxidized intermediates [33,34]. Results from this experiment exhibited that doxepin has a positive increase in its ferric ion reducing ability with increase in its concentration [Fig.2]. The bar diagram below depicts the increase in the ascorbic acid equivalent values ranging from 7.69 ± 3.77 AAE at 25 µg/mL and reaching its maximum to 22.86 ± 5.66 AAE at 500 µg/mL of the drug.

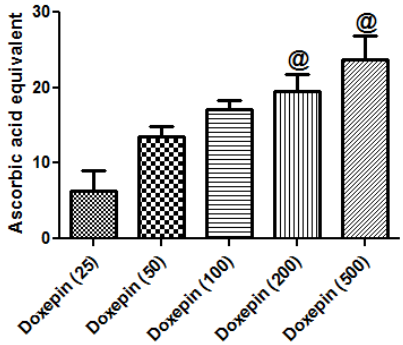

Fig.2 Bar diagram showing the ferric ion reducing activity of doxepin where the X-axis denotes the different concentration of the drug (µg/ml)

*@ means p<0.01, compared to Doxepin(25). The assay was repeated three times (n=3). Values are expressed as mean ± standard deviation (SD).

Estimation of cupric ion reducing capacity: Following the experiment as described by Apak*et al*. [23] doxepin exhibited a positive increase in the reduction of Cu^{2+} ion to Cu^{+} with increasing concentrations of the drug. However, its cupric ion reducing ability is much lower in comparison with other scavenging or reducing abilities, as shown by the observations, ranging from 2.54 ± .75 AAE at 25 µg/mL and reaching its maximum to 6.5 ± 1.11 AAE at 500 µg/mL.

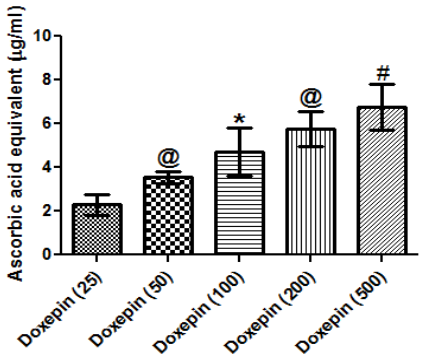

Fig.3 Bar diagram showing the cupric ion reducing activity of doxepin where the X-axis denotes the different concentration of the drug (µg/ml)

 # means p<0.001, compared to Doxepin(25);@ means p<0.01, compared to rest Doxepin(200) and Doxepine(500); means p<0.05, compared to Doxepin(25). The assay was repeated three times (n=3). Values are expressed as mean ± standard deviation (SD).

Estimation of ferrous ion chelating activity: Researchers worldwide considers the ability to chelate transition metal ions like Fe^{2+} to be an important factor in measuring a compound's antioxidant capacity. This is because these ions have the ability to move single electrons which results in the formation and propagation of many radical reactions [35]. Our study shows that the percentage of potential ferrous ion scavenging activity of doxepin varied between 4.88 ± 1.05 % at 25 µg/mL to 35.53 ± 1.55 % at 500 µg/mL[Fig. 4].

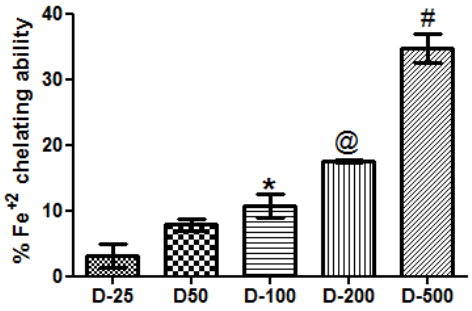

Fig.4 Graphical representation showing the ferrous ion chelating activity of doxepin where the X-axis denotes different concentrations of doxepin (µg/ml)

* # means p<0.001, compared to rest of the concentrations; @ means p<0.01,compared to rest of the concentrations; * means p<0.05, compared to rest of the concentrations.The assay was repeated three times (n=3). Values are expressed as mean ± standard deviation (SD).

Estimation of Hydrogen peroxide scavenging activity: Although hydrogen peroxide itself is a weak oxidizing agent but in physiological systems it can further generate toxic hydroxyl radicals. Results of the experiment performed to determine the hydrogen peroxide scavenging activity of doxepin depicted that in all the concentrations used in the study, the drug showed 20- 30% of scavenging activity. The percentages of scavenging activity calculated were plotted against the different concentrations of doxepin [Fig.5].

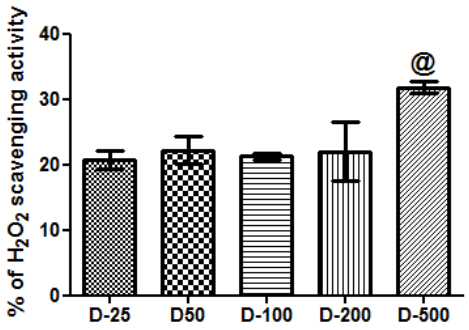

Fig.5 Graphical representation showing the hydrogen peroxide scavenging activity of doxepin where the X-axis denotes different concentrations of doxepin (µg/ml)

* @ means p<0.01, compared to rest of the concentrations.The assay was repeated three times (n=3). Values are expressed as mean ± standard deviation (SD).

Estimation of nitric oxide scavenging activity: As mentioned earlier, researchers worldwide have observed nitric oxide to be a very critical free radical having significant role in generating toxic inflammatory processes in the living system. Our experiment proves that doxepin significantly inhibits nitrite formation by directly competing with oxygen to react with nitric oxide. In all its concentrations the antidepressant showed high levels of scavenging activity ranging from 50-70%.

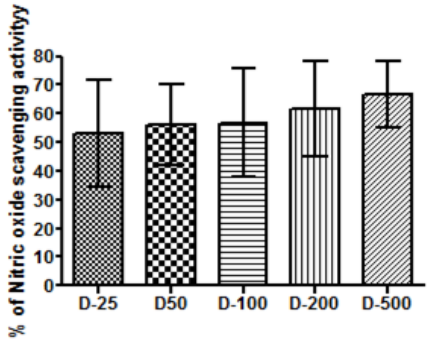

Fig. 6 Graphical representation showing the nitric oxide scavenging activity of doxepin where the X-axis denotes different concentrations of doxepin (µg/ml)

* The assaywas repeated three times (n=3). Values are expressed as mean ± standard deviation (SD).

DISCUSSION

Several investigations during the past few years have pointed out that repeated oxidative stresses often prove to be the major predisposing factor for atherosclerosis, cancer and many other age related neurodegenerative disorders like dementia and Alzheimers disease. However, oxidative stress is usually induced by oxygen as well as nitric oxide radicals. Lien et al showed that non-radical oxygen derivatives, such as, hydrogen peroxide, hypochlorous acid and singlet oxygen may also participate in producing oxidative stress[36]. Certain biochemical reactions induce synthesis of these free radicals leading to structural and functional damages in the proteins, lipids, neurons, nucleic acids and other cellular elements [37].Therefore consumption of antioxidants in some form at a regular basis is now practiced in every community throughout the world. However, most people take antioxidants as manufactured in pharmaceutical industries and also obtain from plants which also contain antioxidants in any of its parts e.g. leaf, stem, flower, fruit and even seeds [16,17,19,38,39]. The present study was designed to search for a novel antioxidant from a known pharmaceutical compound which bears a structural uniformity with a plant derived flavonoid, quercetin, that usually possesses pronouncedantioxidant function. The antidepressant drug doxepin was selected for this purpose and studied experimentally in great details.

Thus a number of standardized tests were designed and performed with doxepin. In phosphomolybdenum assay doxepin showed a constant steady rise in its antioxidant activity with the increase in the amount of the drug keeping ascorbic acid, a known potent antioxidant, as the control [Fig. 1]. An almost identical stepwise increase in the antioxidant action was recorded in doxepin in the ferric ion reducing assay [Fig. 2]. While trying to determine ferrous ion chelating action of the drug it was found that there was a gradual rise in the values from 25 to 200 ug /ml amount followed by a very sharp rise in action with increase in the amount of the agent [Fig. 4]. A gradual increase in values of antioxidant property was noted while testing for cupric ion reducing activity [Fig. 3]. Although this drug revealed presence of antioxidant function while determining its hydrogen peroxide scavenging potency there was hardly any variation in values even though the amount of drug was increased gradually [Fig. 5]. Nitric oxide scavenging property was also detected in doxepin where the percentage of activity was >50 at 25 ug /ml of the compound and did not alter much up to 100ug/ml of the drug. However, the value was a little higher with 200ug/ml while distinctly much higher with 500ug/ml of the compound [Fig. 6].

Oxidation of biomolecules is a routine phenomenon in the human system and hence regular antioxidant rich diet is advised to counteract the process. Although certain chemical compounds like butylhydroxytoluene and butylhydroxyanesole have been claimed to inactivate the process of oxidation these are not prescribed for human consumption due to unavailability of adequate information regarding their stability, safety and carcinogenicity profiles [40,41].

The best examples of non-enzymatic antioxidants are vitamin C and vitamin E which are given routinely to humans throughout the world.

According to Padayatty et al. [42] and Traber et al. [43] regular diet containing these two vitamins help to reduce the risks of cardiovascular diseases, stroke and even cancer. Apart from these two vitamins there are several other antioxidant vitamins which are given to humans on a regular basis; but practically no synthetic antioxidant has been developed by pharmaceutical industries as yet. Green and Ashwood [44] described free radical scavenging property of a few synthetic neuroprotective compounds in experimental animals. One of these compounds tirilazid when given to humans on a trial basis for practical analyses, there was no practical outcome [45]. Thus apart from vitamins there is so far no pharmaceutical compound labeled as an antioxidant for human use. In 2016 Sinha Roy et al[46] observed similar antioxidant action in

another antidepressant drug imipramine.In this background doxepin was selected to determine its potentiality as an antioxidant since this compound is structurally very similar to the known plant derived antioxidant quercetin.

CONCLUSION

Intensive biochemical studies on the pharmaceutical compound doxepin have confirmed it to possess potent antioxidant capacity. The drug doxepin is prescribed for patients suffering from acute mental depression, dementia, anxiety disorders or severe insomnia. Occasionally this drug is tried on patients of chronic ideopathyas the second line of treatment. All such disorders are seen in elderly patients. Therefore if these patients take doxepin in a regular basis they are doubly benefitted in view of its antioxidant function. Presence of antioxidant property in synthetic pharmaceutical compounds has thus opened up a new avenue of routine therapy. The pharmaceutical industries may be advised to label these compounds as antioxidants or alter these structurally to synthesize novel compounds endowed with highly potent antioxidant property.

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