INTRODUCTION

The driving forces to sustain life are some of the processes of biochemical reactions which continue till death of human beings. Such incessant processes in our bodies generate several bio-molecules, of which some are reactive oxygen species (ROSs) and free radicals [1]. Occupational exposure to environmental pollutants such as pesticides, toxic chemical wastes, direct as well as indirect cigarette smoke, gasoline exhaust, urban air pollutants ozone and radiation, and physical stress have been demonstrated to produce similar reactive molecular species in our bodies [2]. These molecules cause oxidative degradation of lipids, proteins, and DNA, activation of procarcinogens, inhibition of cellular and antioxidant defense system, depletion of sulfhydryls, altered calcium homeostasis, changes in gene expression, and induction of abnormal proteins and contribute significantly to human disease pathophysiology [3-5]. Certain substances known as antioxidants act as preventive oxidants caused by such free radicals and ROS. Halliwell [6] defined antioxidants as "any substance that delays, prevents or removes oxidative damage to a target molecule." The antioxidant vitamins such as vitamins C and E, ß-carotene and proanthocyanidins, antioxidant minerals such as zinc and selenium, and antioxidant enzymes such as glutathione, superoxide dismutase, and catalase, have been vividly studied for their potential role in the prevention of degenerative diseases including tumor growth and carcinogenesis [5-8]. Research evidence has suggested a link between increased levels of ROS and disturbed activities of enzymatic and nonenzymatic antioxidants in diseases associated with aging [9].

Through several studies, the different modes of action of various antioxidants have been observed: As preventers of oxidation reactions caused by free radicals, free lipid radical formation inhibitors, by breaking the chain of auto-oxidation reactions, as quenchers of singlet oxygen, by converting the hydroperoxides through reduction and the metal pro-oxidants by chelating metals into stable compounds, and finally as inhibitors of pro-oxidative enzymes [10-14].

Flavonoids are a class of naturally occurring polyphenolic compounds, isolated from a wide range of vascular plants. They generally occur in plants as glycosylated derivatives, and they contribute to the brilliant shades of blue, scarlet, and orange, in leaves, flowers, and fruits. Apart from various vegetables and fruits, flavonoids are found in seeds, nuts, grains, spices, and different medicinal plants as well in beverages, such as wine particularly red wine, tea, and at lower levels in beer [15]. The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings. Different biological activities of flavonoids had been the subject of several studies in the past years, and it has been revealed that they exhibit antimicrobial, antiinflammatory, antiviral, anti-inflammatory, and vasodilating actions. However, most interests have been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals [16].

Among the flavonoid subclass of flavonoids, quercetin is a major representative. Quercetin is the flavonoid widely distributed in nature and is one of the most abundant dietary flavonoids with an average...
daily consumption of 25-50 mg. Quercetin has been observed to act mainly in the prevention of oxidation of low-density lipoproteins by scavenging free radicals and chelating transition metal ions [17].

A large number of studies by different workers from several parts of the world have repeatedly provided enough evidence that pharmaceutical compounds synthesized for a particular pharmacological action often simultaneously possess some other function equally efficiently [18-21].

Such observations have repetitively proved that multiplicity of function in chemically synthesized drugs is a rule rather than an exception. With this background, the structural similarity of the synthetic antidepressant compound doxepin with the known antioxidant quercetin has replenished sufficient evidence to explore antioxidant action in this drug. Moreover, there is hardly any synthetic antidepressant available in the market for human consumption except the vitamins. The present study was therefore designed to carry out an elaborate experimental work to prove the efficacy of doxepin as an antioxidant with the help of a number of known standard tests.

**METHODS**

**Drug**

Doxepin was obtained as pure dry powder from Sigma-Aldrich, USA. It was preserved at 4°C. The drug powder was soluble in water.

**Chemicals and reagents**

All the chemicals and reagents used in the following different assays were purchased from Loba Chemie (Mumbai, India), DCM Chemical Laboratory (SRL, Mumbai, India), Merck (Bangalore, India), and from HiMedia (Mumbai, India). They were all of analytical grade.

**Preparation of the sample**

For every analysis, a freshly prepared working solution of 1 mg/ml of the drug powder was prepared by dissolving in Milli-Q grade water.

**Protocols for detection of in vitro antioxidant and free radical scavenging activity**

**Antioxidant capacity determination**

The phosphomolybdenum method as described by Prieto et al. [22] was followed for the estimation of total antioxidant capacity. This assay is 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. Tubes containing 0.2 ml aliquots of concentrations 25, 50, 100, 200, and 500 µg/ml of the drug solution were mixed with 1.8 ml of distilled water and 2 ml of phosphomolybdic reagent (0.31 M sulfuric acid, 2 mM sodium phosphate, and 4 mM ammonium molybdate). The amount of 0.2 ml of water was used instead of doxepinas the control. All of the tubes were then incubated in a water bath set at 95°C for 90 minutes. After the completion of the reaction, absorbances were measured at 695 nm using a ultraviolet-visible (UV-VIS) spectrophotometer (Evolution 201, Thermo Scientific) against the blank. A standard curve was prepared using different concentrations of ascorbic acid (1000, 500, 250, 125, 62.5, and 31.25 µg/ml). The total antioxidant activity is expressed as the µg/ml equivalents of ascorbic acid, defined as ascorbic acid equivalents activity and calculated as % of inhibition=(A

**Ferric ion reducing capacity determination**

This assay was performed by following the method as described by Prieto et al. [22]. Accordingly, 0.1 ml aliquots of different concentration of the sample solution (25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml) were taken in different tubes and to each one of them 0.5 ml of deionized water and 0.09 ml of 95% ethanol were added. Subsequently, further addition of each of 0.15 ml each of 1 M HCl, 1% potassium ferrocyanide, 0.05 ml of 1% sodium dodecyl sulfate, and 0.1 ml of 0.2% ferric chloride were followed. The tubes were then vortexed properly, and readings were taken at 700 nm in a spectrophotometer (Evolution 201, Thermo Fisher) against the blank. Potent antioxidants reduce Fe³⁺ ions to Fe²⁺ which forms a Prussian blue colored complex with the ferrocyanide. The intensity of the Prussian blue color in the reaction tubes as measured at the said wavelength monitors the amount of the Fe³⁺ ions generated. Thus, a higher ferric ion reducing power was indicated by a greater absorbance. A standard curve was prepared by different concentrations of ascorbic acid (1000, 500, 250, 125, 62.5 and 31.25 µg/ml).

**Cupric ion reducing capacity determination**

For this assay, the protocol as described by Apak et al. [23] was followed. The basic principle is based on the measurement of absorbance at 450 nm by the formation of stable colored complex between neocuproine and copper (I). Copper (II)-neocuproine (Cu [II]-Nc) reagent is utilized as the oxidizing agent for the reaction. As for the purpose, 0.6 ml volume each of double distilled water, cupric chloride (0.01 M), and sodium citrate (25% w/v) were added to correctly labeled tubes and shaken vigorously for proper mixing. This was followed by the addition of 0.6 ml volume of sample solution in increasing concentrations (25, 50, 100, 200, and 500 µg/ml) to each of the respective tubes. Finally, 0.6 ml neocuproine (0.0075 M) was added to all of them and then the reaction mixtures were incubated at room temperature for 30 minutes. Absorbances were taken at 450 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank. A standard curve was prepared by different concentrations of ascorbic acid (1000, 500, 250, 125, 62.5, and 31.25 µg/ml). The results are expressed as µg/ml equivalents of ascorbic acid.

**Ferrous ion chelating activity determination**

The standard method as described by [24] was followed for the estimation of ferrous ion chelating ability of the drug doxepin. The basic principle of this assay is that ferrozine, which initiates the reaction by combining with divalent iron to form a stable magenta complex species, the absorbance of which is measured at 562 nm. In the presence of potent metal ion chelators, the ferrozine-Fe²⁺ complex formation is interrupted as they compete with ferrozine in chelating the ferrous ions. Briefly, to 3 ml of different concentrations of the drug solution (25, 50, 100, 200, 500 µg/ml) 0.15 ml of 2 mM FeCl₃ was added. The reaction was then initiated by the addition of 0.3 ml of 5 mM ferrozine into the mixture and incubated at room temperature for 10 minutes. Finally, the absorbance of the ferrozine-ferrous ion complex was read at 562 nm against the blank. Ethylene diamino tetra acetate was used as the positive control for this assay. The chelating activity of the drug was calculated as ferrous ion chelating ability in %=[1-(test sample absorbance/blank sample absorbance)]×100%.

**Hydrogen peroxide scavenging activity determination**

Although hydrogen peroxide is not a free radical, in physiological system, it forms complexes with metals such as iron and copper and reacts to produce highly reactive hydroxyl radicals which are extremely toxic to the living tissues [25]. According to the modified method of determining hydrogen peroxide scavenging activity by Mukhopadhyay et al. [26], 1.5 ml of different concentrations of the drug solution (25, 50, 100, 200, 500 µg/ml) were added to 0.25 ml of 1 mM ferrous ammonium sulfate and mixed and then to each tube, 6.25 µL of 5 mM hydrogen peroxide were added, and all of them were then incubated for 5 minutes at room temperature in dark. Following the incubation, 1.5 ml volume of 1,10-phenanthroline monohydrate was pipetted into the reaction mixtures and again kept for 1 minute in dark at room temperature. After that, absorbances were measured at 510 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank. Ascorbic acid was used as the reference standard of the assay. The percentage of hydrogen peroxide scavenging activity of the drug was calculated as given below:

% of scavenging activity=[A

Where A

is the absorbance of solution containing only ferrous ammonium sulfate and 1,10-phenanthroline and A

is the absorbance
of the solution containing ferrous ammonium sulfate, hydrogen peroxide, 1,10-phenanthroline and along with test compound having expected hydrogen peroxide scavenging activity.

**Nitric oxide scavenging activity determination**

Chronic physiological generation of nitric oxide leads to a number of significant toxic effects including vascular collapse with septic shock, and it also plays a key role in inflammatory conditions such as juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis [27]. For the assay to estimate the nitric oxide scavenging activity of antioxidants, the method as described by Chaudhuri et al. [28] was followed. Here, sodium nitroprusside was used as the nitric oxide donor, which when generated in the system interacts with oxygen to form the highly reactive peroxynitrite anion (ONOO−), and this can be measured by Griess-Illosvoy reaction [29]. As per the protocol, 1 ml of different concentrations of doxepin (25, 50, 100, 200, and 500 µg/ml) was added in tubes to which 1 ml of 10 mM sodium nitroprusside was given and mixed properly. This was followed by incubation for 3 hrs at room temperature. After this, 1 ml of Griess reagent A (1% w/v sulfanilamide) was pipetted into each of the tubes, and again they were incubated for 20 minutes at 30°C. Finally, the assay is completed with the addition of 1 ml of Griess reagent B (0.1% w/v naphthylethylenediamine dihydrochloride or NED) to the mixture tubes and again incubated for 10 minutes in dark at room temperature. Absorbances were recorded at 546 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank. Ascorbic acid was used as the positive control. The percentage of nitric oxide radical scavenging activity of the drug solutions was calculated as given below:

\[
\% \text{ of scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100
\]

**Statistical analyses**

Statistical analysis was done using One way Anova following Bonferroni post hoc test (GraphPad Prism version 5.0 software).

**RESULTS**

**Determination of total antioxidant activity by phosphomolybdenum assay**

Our result indicates an increasing trend of total antioxidant activity with increase in concentration of the antidepressant compound ranging from 25 µg/ml to 500 µg/ml. The obtained average angular error (AAE) values have been depicted in the graph (Fig. 1) against the different concentrations of doxepin. From the bar diagram, it can be observed that doxepin has a moderately high molybdenum ion reducing ability with the values ranging from 25.3±1.47 AAE at 25 µg/ml and reaching its maximum to 174.35±8.39 AAE at 500 µg/ml.

**Detection of ferric ion reducing activity in doxepin**

Researchers worldwide have studied that compounds which have potent reducing power can act as electron donors and also can reduce the oxidized intermediates. This is a measure of significant antioxidant potentiality of a compound [30,31]. Observations from this experiment indicate that there is a positive increase in the ferric ion reducing ability of doxepin with its increasing concentration (Fig. 2). The ascorbic acid equivalent values ranged 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.

**Evaluation of cupric ion reducing activity**

Molecules having antioxidant capacities, specially belonging to the class of hydroxyl radicals of phenolic compounds, have the ability to reduce Cu²⁺ ions to Cu¹⁺. These Cu¹⁺ ions generated in the system, then react and form the colored complex with neocuproine whose absorbance is measured at the said wavelength [23]. Doxepin exhibited increased reduction of Cu²⁺ ion to Cu¹⁺ 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±0.75 AAE at 25 µg/ml, and reaching its maximum to 6.5±1.11 AAE at 500 µg/ml.

**Action of doxepin on ferrous ion chelating property**

The chelating activity of the drug was estimated on the basis of the measurement of color reduction of the ferrozine - Fe²⁺ complex. As transition metal ions such as Fe²⁺ can move single electrons which result in the formation and propagation of many radical reactions, the ability to chelate them is considered to be a significant measurement of a compound’s antioxidant capacity [32]. Our study shows that doxepin was able to potentially chelate the metal ion which increased in a dose-dependent manner. The percentage of scavenging activity was calculated and plotted against the different concentrations of the drug (Fig. 3). The values of the activity percentage varied between 4.88±1.05% at 25 µg/ml and 35.53±1.55% at 500 µg/ml.
Effect of doxepin on hydrogen peroxide scavenging property

As mentioned earlier, hydrogen peroxide although a weak oxidizing agent, but it can further generate toxic hydroxyl radical in living systems. Following the protocol as described by Mukhopadhy et al. [26], the percentage of hydrogen peroxide scavenging activity of doxepin was calculated and it was plotted against the different concentrations of the drug (Fig. 4). As from the figure, it can be observed that in all the concentrations used in the study, doxepin showed 20-30% of hydrogen peroxide scavenging activity.

Determination of nitric oxide scavenging activity of doxepin

Studies from several researches have revealed that nitric oxide is an important free radical which has a key role in generating toxic inflammatory processes in the physiological system. Our experiment proves that doxepin significantly inhibits nitrite formation by directly competing with oxygen to react with nitric oxide. As observed from Fig. 5, in all the concentrations, doxepin showed high levels of nitric oxide scavenging activity ranging from 50% to 70%.

DISCUSSION

Recent scientific investigations throughout the world suggest that there is strong evidence of involvement of oxidative stress in the pathogenesis of cancer, atherosclerosis, inflammatory diseases, and neurodegenerative disorders such as Alzheimer, Parkinsonism, and convalescent. Such an oxidative stress is primarily induced by the RO which include both oxygen radical and nitric oxide radicals. However, certain non-radical derivatives of oxygen like hydrogen peroxide, hypochlorous acid, and singlet oxygen can also be involved in this process [33]. These free radicals synthesized by various biochemical reactions in the human body can cause structural and functional damage to the neurons, proteins, lipids, nucleic acids, and different cellular molecules [34]. Thus, there is an urgent need for the search of free radical scavengers or antioxidants for the prevention of such cellular damages [35]. Although most of the studies are focused either on synthesized new molecules in pharmaceutical industries or detection of antioxidants in plant products, this study has been directed to search for antioxidants among known pharmacological compounds with structural similarity with flavonoids of plant origin which possess
potent antioxidant properties [36,37]. The antidepressant drug doxepin was found to resemble structurally the plant-derived flavonoid compound quercetin.

While determining the total antioxidant activity in doxepin by phosphomolybdenum assay, it was found that there was a steady, gradual rise in the activity with the increase in the amount of the compound with respect to amino acid equivalent (Fig. 1). Again in the ferric reducing antioxidant power assay, doxepin distinctly revealed a stepwise increase in antioxidant property much like the control (Fig. 2). Subsequently by the cupric reducing antioxidant capacity assay process, doxepin showed gradually increasing reduction of Cu2+ ion to Cu+ with elevation in the amount of doxepin (Fig. 6). In a separate study, doxepin showed definite Fe3+ chelating action whose values increased in a gradual manner from 25 to 200 µg/ml concentration, however, with 500 µg/ml of the drug there was a very sharp rise in the values (Fig. 3). Doxepin although had shown hydrogen peroxide scavenging function, the values hardly varied between the amounts of 25 and 200 µg/ml, a distinct rise in action was observed with 500 µg/ml of the drug (Fig. 4). Finally, doxepin revealed nitric oxide scavenging property, the percentage activity was >50 at 25 µg/ml of doxepin and remained almost the same up to 100 µg/ml of the drug. However, the values were slightly higher with 200 µg/ml of doxepin but sharply higher with 500 µg/ml of the compound.

Free radicals are known to cause oxidation of biomolecules in the human system, and antioxidant-rich diets can protect the body from free radicals [38]. Synthetic antioxidants such as butylhydroxyanisole or butylhydroxytoluene have been used to decelerate such processes. However, due to their unstable and volatile nature, scarcity of information regarding their safety, stability, and carcinogenicity these chemical compounds are still not in use in health-care systems [38,39]. Non-enzymatic antioxidants and antioxidant enzymes counteract the effects of ROS and RNS leading to the risk of oxidative stress [40].

The non-enzymatic antioxidants include the vitamins of which vitamin C and vitamin E are most promising agents and are in regular use. Epidemiological studies have shown that diets containing these vitamins help reduce risks of cardiovascular disease, stroke, and even cancer [41,42]. Both of these vitamins along with several other antioxidant vitamins are available in the market for human consumption, but there is practically no synthetic compound that can act as antioxidants. There are only a few reports on the detection of antioxidants property in synthesized chemical compounds. In 2005, Green and Ashwood reported on free radical scavenging activity of several synthetic compounds for neuroprotection in stroke in experimental animals [43]. However, preclinical studies with such compounds were neither comprehensive nor consistent. One of these compounds, tirilazad was investigated by some researchers but human trials demonstrated no effect on mortality or other outcomes in several disorders [44]. Thus, in view of inadequacy of information on possibilities of developing or obtaining synthesized antioxidants other than the vitamins doxepin was selected to determine its antioxidant potentiality on the basis of its structural similarity with quercetin, the known highly active antioxidant.

Doxepin is a pharmaceutical compound which is primarily given to patients suffering from acute mental depression and anxiety disorders. Doxepin is sometimes prescribed for patients complaining about insomnia as well. However, it has also been found to treat chronic idiopathy as the second line of treatment. Most of the above disorders are found to occur among elderly people, and doxepin is administered on a regular basis for long periods. Therefore, the patients who are on a regular intake of doxepin are likely to be benefitted to have an antioxidant simultaneously. A recent study has proved the efficacy of the antidepressant antipsychotic agent imipramine to process antioxidant properties also [45].

Therefore, the concept of the presence of multiplicity of action among pharmacological compounds is being proved again. Much like imipramine, toxicity profile and human tolerance levels of doxepin are well documented. Therefore, both these two drugs can be developed further by structural modifications to produce highly active antioxidants that can be safely administered to patients of all ages and also those who have greater chances of acquiring cancer, atherosclerosis, neurodegenerative diseases, and many other serious disorders. Therefore, such investigations are likely to open up new avenues of research on detection and determination of possible antioxidants among known pharmaceutical compounds.

CONCLUSION

Human body possesses a complex system of natural enzymatic and non-enzymatic antioxidant defense mechanisms that can counteract moderate to highly toxic effects of free radicals and other oxidants that are responsible for many human diseases including cancer. In view of the necessity to discover new active antioxidants, a search was directed to find out such agents from already known pharmacological compounds. On the basis of information of the presence of powerful antioxidant function in naturally occurring flavonoids in plants, the structurally similar compound doxepin was selected, and intensive studies have confirmed doxepin to be a highly potent antioxidant. In this way, novel antioxidants can be discovered from already known pharmaceutical compounds that possess structural similarity with known antioxidants including flavonoids.

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