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EXPERIMENTAL ANALYSES OF ANTIOXIDANT CAPACITY OF A COMMON ANTIDEPRESSANT DRUG

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ABSTRACT
Severe damages may be produced in the bio-molecules of human system by free radicals that may ultimately lead to cardiovascular, neurodegenerative and autoimmune diseases. The adverse effects of free radicals may be reduced to a great extent by synthetic and naturally available antioxidants. The flavonoid quercetin present in many plants has been identified as a potent antioxidant. The pharmaceutical compound imipramine, prescribed for patients suffering from depression and psychosis, is structurally similar to quercetin and was therefore selected to determine its antioxidant potentiality. For this purpose highly recognised and standardised procedures were followed. In the FRAP assay and phosphomolybdenum assay imipramine revealed much greater antioxidant value than the control ascorbic acid. In CUPRAC assay and in determination of ferrous ion chelating property imipramine produced positive antioxidant capacity in a gradually increasing dose dependent manner, although the values were less than the control. Imipramine failed to show nitric oxide scavenging activity. This study opens up an important positive side of therapy for patients suffering from depression or psychosis, who have to take imipramine on a regular basis for a long period of time. The antioxidant property of imipramine would most likely place such patients in a much more advantageous position.

KEYWORDS: Imipramine, Flavonoid, Antidepressant, Quercetin, Antioxidant, Reactive Oxygen Species.

INTRODUCTION
Oxidation may be defined as the transfer of an electron from electron rich to electron deficient entity. Metals and metallic compounds are considered as natural oxidizing agents as they are present naturally in all ecosystems.[1] Such heavy metals possess toxigenic and carcinogenic potentialities. Interaction of these heavy metals with deoxyribonucleic acid (DNA) and proteins often results in denaturation of biological macromolecules.[2] Several heavy metals like lead, arsenic, mercury, iron, cadmium, chromium and cobalt are capable of producing reactive free radical species that may finally terminate in lipid peroxidation and oxidation of DNA and ribonucleic acid (RNA).[3] These oxidative processes can be slowed down or even prevented by many agents known as antioxidants. Antioxidants can trap the free radicals thereby terminating a series of reactions by arresting the process of free radical generation or by chelating the respective metal ions and also due to suppression of reaction of biomolecules with the Reactive Oxygen Species (ROS) by direct scavenging of free radicals. Although Halliwell (1996).[4] observed *in vivo* that non-enzymatic antioxidants and antioxidant enzymes can both neutralise the harmful effects of ROS, antioxidative defence system in healthy humans can efficiently balance generation of free radicals; but individuals suffering from different diseases that favour generation of free radicals show oxidative stress due to decrease in the antioxidant levels.[5] Antioxidants may be divided into two groups enzymatic and non-enzymatic. Flavonoids are regarded as non-enzymatic antioxidants apart from the vitamins C, E and the carotenoids, since flavonoids can offer protection against oxidative stress.[6] Flavonoids are polyphenolic compounds possessing 15 carbon atoms, two benzene rings which are joined by a linear three carbon atom chain. Many fruits vegetables, olive oil, tea and red wine are known to contain the flavonoid quercetin.[7] Presence of quercetin in different parts of plants has been reported by several researchers during the past few years.[8-13] Arsenic induced oxidation in experimental animals could be fully driven out by quercetin.[14] In this background the antidepressant drug imipramine was selected to determine its antioxidant potentiality since imipramine possesses a close structural similarity with quarcetin.

MATERIALS AND METHODS

Chemicals

Different solvents and reagents used in this study were purchased from various well known manufacturers in India as pure chemicals in dry powder form. All the chemicals and reagents used in this study were of analytical grade and were preserved according to the stipulated instructions of the respective manufacturers.

Preparation of standard solution of the drug for antioxidant assay

1 mg/ml of the stock solution of the drug imipramine was prepared for the assay by dissolving a suitable amount in Milli-Q grade water. This was preserved at 4°C. Different concentrations of working standards of imipramine (25, 50,100, 200, 500 µg/ml) were freshly prepared from that stock solution whenever needed.

Determination of antioxidant property of imipramine with the help of FRAP assay

Ferric ion reducing power assay as described by Prieto *et al* [15] was followed with some modifications. In this process an antioxidant compound is able to reduce Fe3+ to Fe2+ by donating an electron. This method measures the ability of an antioxidant in reducing ferric ion. This ion then gets conjugated with the ferricyanide ion to form a Prussian blue coloured product at low pH, which can be spectrophotometrically measured at 700 nm. The presence of sodium dodecyl sulphate (SDS) prevents the formation of turbidity in the solution.

Fe^{+3} + Antioxidant = Fe^{+2} + Oxidized antioxidant

Fe^{+2} + $Fe(CN)_6^{3-}$ = $Fe[Fe(CN)_6]$-(Prussian blue coloured product)

For this purpose 2 ml centrifuge tubes were taken and labelled for appropriate concentrations. 0.1 ml volume of increasing concentrations of imipramine (25, 50, 100, 200,500 µg/ml) were added to 0.5 ml of deinonized water and 0.09 ml of 95% ethanol. Subsequently, 0.15 ml each of 1M HCl and 1% (w/v) potassium ferricyanide were also added to the reaction mixture, followed by further addition of 0.05 ml of 1% (w/v) SDS, 0.1 ml of 0.2% (w/v) ferric chloride. Then all the tubes were vortexed and incubated at 50°C in a water bath for 20 minutes. During this period, the UV-VIS spectrophotometer (Evolution 201, Thermo Fisher) was set at 700 nm. After incubation in water bath, tubes were taken out and the lids of the tubes were opened for a rapid cooling. Then readings were taken at 700 nm. By this process the amount of Fe2+ could be examined by measuring the formation of Prussian blue colour. The antioxidant capacity of imipramine was evaluated from the standard curve of ascorbic acid concentrations against O.D. at 700 nm. Ascorbic acid was used as the positive reference standard. All assays were run in triplicates and averaged. The reducing ability of imipramine was directly proportional to the absorbance reading i.e. a higher reducing power was indicated by a greater absorbance. A standard curve was prepared with the help of different concentrations (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml) of ascorbic acid.

The ferric ion reducing activity is expressed as the µg/mL equivalent of ascorbic acid, defined as ascorbic acid equivalent activity (AAEA) and calculated as : % 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 different drug solutions. These values were then plotted against different concentrations of the drug used in this assay.

Determination of total antioxidant capacity by phosphomolybdenum assay

The total antioxidant capacity of imipramine was estimated with the help of phosphomolybdenum assay procedure as described by Prieto *et al*.[15] The principle of this assay is the reduction of Mo (VI) to Mo (V) by an agent and consequent formation of a green phosphate/ Mo (V) complex at acidic pH. This coloured compound has its absorbance at 695 nm. Increase in absorbance of the reaction mixture indicate the increase in antioxidant activity.

In this process tubes containing 0.2 ml of imipramine at varying concentrations (25, 50, 100, 200, 500 µg/ml) were mixed with 1.8 ml of distilled water and subsequently 2 ml of phosphomolybdenum reagent (0.31 M sulphuric acid, 2 mM sodium phosphate plus 4 mM ammonium molybdate were taken) in previously labeled centrifuge tubes. For the control 0.2 mL of water was used in place of imipramine. The tubes were then kept at 95°C in a water bath for 90 minutes. The mixtures were cooled to room temperature and readings were taken at 695 nm in the same UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against blank. Ascorbic acid was used as the positive reference standard. All assays were run in triplicates and averaged. The total antioxidant capacity of imipramine was obtained from the standard curve of ascorbic acid concentrations against O.D. at 695 nm. A standard curve was prepared by different concentrations of ascorbic acid (3.125 µg/ml- 200 µg/ml). Calculations were made as given earlier.

Determination of copper ion reducing antioxidant power (CUPRAC assay)

This assay was performed using a method illustrated by Apak *et al*.[16] In this assay specialy the hydroxyl groups of phenolic compounds are changed to quinines [oxidized form] in the presence of cupric chloride (Cu+2) which is then reduced to Cu+. The formed Cu+ then reacts with the chromogen, neocuproine (2, 9 dimethyl-1,10 phenanthroline) to form a yellow coloured complex which can be spectrophotometrically measured at 450 nm.

For this purpose to each of the labelled centrifuge tubes was added 0.6 ml of distilled water, 0.01 M of cupric chloride and 25% solution of sodium citrate one by one

and mixed thoroughly. Then 0.6 ml of increasing amounts of imipramine (25, 50, 100, 200, 500 µg/ml) were added to the tubes followed by proper vortexing. Finally to each tube 0.6 ml neocuproine (0.0075M) was added, vortexed and the reaction mixtures were then incubated at room temperature for 30 minutes. Readings were taken in the same spectrophotometer (Evolution 201, Thermo Scientific) at 450 nm. The antioxidant capacity of imipramine was evaluated from the standard curve of ascorbic acid concentrations against O.D. at 450 nm. Ascorbic acid was used as the positive reference standard. All assays were run in triplicates and averaged. A standard curve was prepared with different concentrations (0.5 mg/ml, 0.4 mg/ml, 0.3 mg/ml) of ascorbic acid as described.

Ferrous ion chelating activity determination in imipramine

The standard method as described by Dinis *et al*.[17] was followed for the estimation of ferrous ion chelating ability of the drug imipramine. The basic principle of this assay is that ferrozine [FZ, disodium salt of 3-(2-pyridyl)-5,-bis(4-phenylsulphonic acid)-1,2,4-triazine], 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 antioxidants in the system they would efficiently chelate the ferrous ions and thus impede the formation of the ferrous-ferrozine complex. This would cause a decrease in the intensity of colour of the reaction mixture.

Briefly, to 3 mL of different concentrations of the drug solution (25, 50, 100, 200, 500 µg/ml), 0.15 mL of 2 mM $FeCl_2$ was added. The reaction was initiated by addition of 0.3 mL of 5 mM ferrozine into the mixture which was then 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 (EDTA) was used as the positive control for this assay.

The chelating activity of the drug was calculated as –
Ferrous ion chelating ability in % = [1-(drug sample absorbance/blank sample absorbance)] X 100%. The percentage chelating activity values were then plotted against different concentrations of the drug used in this assay.

Determination of nitric oxide radical scavenging activity

To estimate nitric oxide scavenging activity of imipramine, the method as described by Chaudhuri *et al*.[2] was followed. Griess test, as it is termed, is a chemical analysis test which detects presence of organic nitrite compounds. The Griess diazotization reaction on which the Griess reagent relies was first described in 1958 by Peter Griess. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of Griess reaction. Griess reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled with NED to form the chromophoric azo-derivative which absorbs light at 546 nm. Herein, a measure in the decrease in absorbance at that particular wavelength was indicated as the increased nitric oxide scavenging ability of the antioxidant.

As per the protocol, 1 mL of different concentrations of imipramine (25, 50, 100, 200, 500 µg/ml) was taken in tubes to which 1 mL of 10 mM sodium nitroprusside was added and mixed thoroughly. This was followed by incubation for 3 hours at room temperature. After this, 1 mL of Griess reagent A (1% w/v sulphanilamide) was pipetted into each of the tubes and again they were incubated for 20 minutes at 30°C. Finally, the test was completed with the addition of 1 mL of Griess reagent B (0.1% w/v NED) to the mixture tubes followed by incubation for 10 minutes in dark at room temperature. Absorbance was recorded at 546 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against the blank.

Percentage of nitric oxide radical scavenging activity of the drug solutions was calculated as: % of scavenging activity = (Absorbance of control – Absorbance of test/ Absorbance of control) X 100. The respective values were then plotted against different concentrations of imipramine used in this experiment.

Statistical analysis

Results were given as mean ± standard deviation of 3 replicates. The results were analyzed statistically using Graph-pad prism version 5.0 software (Graph Pad Software Inc., La Jolla, CA, USA).

RESULTS

Determination of antioxidant property of antidepressant drug imipramine with the help of ferric ion reducing property

FRAP measures the significant reducing potency of imipramine. Since an antioxidant can reduce Fe3+ to Fe2+ by donating an electron, the resultant Fe2+ forms a complex with ferrocyanide that was monitored by measuring the formation of Prussian blue coloured complex which absorbed light at 700 nm. Our study revealed that the reducing power of imipramine increased with the increase of their concentrations ranging from 25µg/ ml to 500 µg/ml (Figure 1) From the graph it can be observed that imipramine had a significant ferric ion reducing ability with the values ranging from 86.82± 9.64 AAE at 25 µg/mL and reaching its maximum to 716.4± 177.1 AAE at 500 µg/ml (Table 1). The values proved beyond doubt that imipramine is possesses greater antioxidant property than ascorbic acid.

Estimation of antioxidant property by phosphomolybdenum assay

In this assay our study confirmed that the antioxidant capacity of imipramine increased in a dose dependent manner ranging from 25µg/ml to 500 µg/ml (Figure 2).The ascorbic acid equivalent values ranged from 70.70±22.74 at 25 µg/mL and reaching its maximum to 727.3±67.45 at 500 µg/mL of the drug (Table 2). The obtained AAE value proved that imipramine is a better antioxidant than ascorbic acid and moreover, this assay showed better molybdenum ion reducing power than ferric ion reducing power.

Detection of Cupric ion reducing activity of imipramine

Our study revealed that the reducing power of imipramine although increased with the increase in their concentrations ranging from 25µg/ml to 500 µg/ml, (figure 3) it was less active than the well known antioxidant ascorbic acid as shown by the observations, ranging from 22.08± 0.98 AAE at 25 µg/mL and reaching its maximum to 10.42± 1.2 AAE at 500 µg/mL (Table 3).

Effect of imipramine on ferrous ion chelating activity

The chelating activity of the drug was estimated on the basis of the measurement of colour reduction of the ferrozine – Fe^{2+} complex. As transition metal ions like Fe^{2+} has the ability to move single electrons which results 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 [18]. Our study showed that imipramine 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 different concentrations of the drug. The values of the activity percentage varied between 4.88 + 1.05 % at 25µg/mL and 35.53 + 1.55 % at 500 µg/mL (Figure 4).

Determination of nitric oxide scavenging property of imipramine

It was observed that different concentrations of imipramine were not able to scavenge the nitric oxide radicals when compared with ascorbic acid, which served as the positive control.

Table 1: Ascorbic acid equivalent values of increasing amounts of imipramine by FRAP assay.

Concentration of imipramine(µg/ml)	*Ascorbic acid equivalent (AAE) value (µg/ml)
25	86.82± 9.64
50	157.9± 37.85
100	256.0± 59.87
200	425.5± 102.1
500	716.4± 177.1

*Values are Mean ± SD

Table 2: Ascorbic acid equivalent values of increasing amounts of imipramine by Phosphomolybdenum assay.

Concentration of imipramine(µg/ml)	*Ascorbic acid equivalent (AAE) value (µg/ml)
25	70.70±22.74
50	118.9±16.27
100	216.00±25.60
200	382.6±47.11
500	727.3±67.45

*Values are Mean ± SD

Table 3: Ascorbic acid equivalent values of increasing amounts of imipramine by cupric ion reducing assay.

Concentration of imipramine(µg/ml)	*Ascorbic acid equivalent value ((µg/ml)
25	2.08± 0.98
50	2.77± 0.98
100	5.14±1.03
200	7.06± 0.80
500	10.42± 1.2

*Values are Mean ± SD

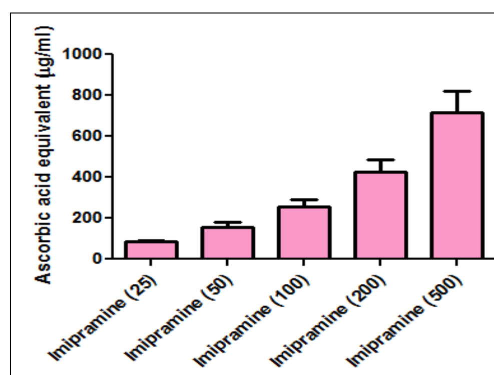

Fig 1: Bar diagram represents the determination of ferric ion reducing antioxidant capacity of imipramine by FRAP assay. X axis indicates concentration of imipramine and Y axis is plotted against concentration of ascorbic acid.

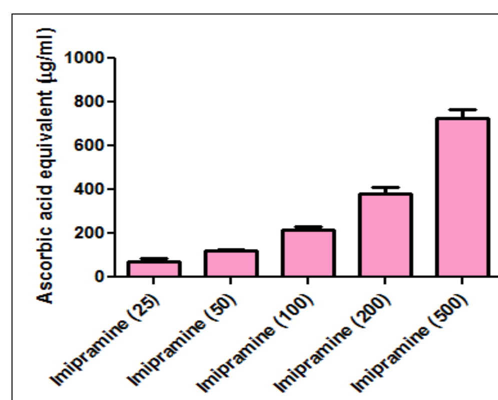

Fig 2: Bar diagram represents the determination of total antioxidant capacity of imipramine by Phosphomolybdenum assay. X axis indicates concentration of imipramine and Y axis is plotted against concentration of ascorbic acid.

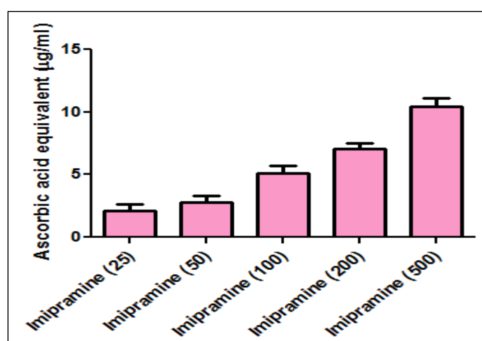

Fig 3: Bar diagram represents the determination Cupric ion reducing activity of imipramine by CUPRAC assay. X axis indicates concentration of imipramine and Y axis is plotted against concentration of ascorbic acid.

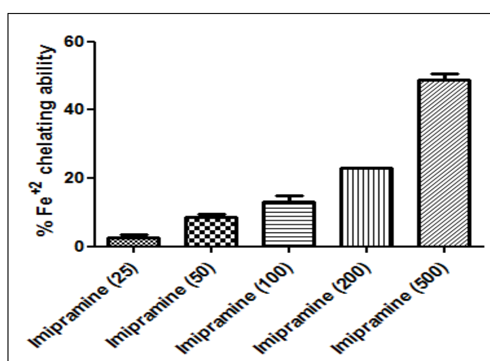

Fig 4: Graphical representation showing the ferrous ion chelating activity of imipramine where the X-axis denotes different concentrations of imipramine (µg/ml).

DISCUSSION

The antidepressant drug imipramine was found to possess potent antioxidant action. The antioxidant capacity of imipramine was distinctly higher than the competent antioxidant ascorbic acid in FRAP assay and phosphomolymdenum assay, where ascorbic acid served as positive control in both the assays; however, in the CUPRAC assay system imipramine was less active than ascorbic acid although the reducing power of imipramine gradually increased with increasing amounts of the compound. In a separate study imipramine showed definite Fe^{2+} chelating action whose values increased in a gradual manner from 25 to 200 µg/mL concentration, but with 500 µg/mL of the drug there was a very sharp rise in the values, where EDTA served as the positive control. Nitric oxide is an important free radical which has a key role in generating toxic inflammatory processes in the physiological system. Our observations revealed that different concentrations of imipramine were not able to scavenge the nitric oxide radicals at all in comparison with ascorbic acid, which served as the positive control.

Imipramine is structurally similar to the flavonoid quarcetin, whose antioxidant capacity has been repeatedly observed by various researchers.[8-13] In flavonoids the hydroxyl group is substituted by flavan moiety. This particular characteristic feature is essential to scavenge free radicals and prevent oxidation of biological molecules by conversion of ROS into inactive forms.[19] Since imipramine possesses structural similarity with flavonoids its antioxidant property would possibly be very useful in the prevention and treatment of oxidative stress induced diseases like dementia, alzheimer's disease, Parkinson's disease and Huntington's disease.

The drug imipramine is given to patients suffering from severe depression which is associated often with agitation and anxiety. It is also used in the treatment of enuresis. Such disorders are seen among elderly persons. These patients receive imipramine for a long period of time. Therefore the patients who are given such a unique drug are predominantly benefited since the present study proves beyond doubt that imipramine is a highly powerful antioxidant. Imipramine is routinely used not only as an antidepressant but also as an antipsychotic agent and hence its entire pharmacological parameters including its toxicity profile are known. In this background pharmaceutical industries are certainly in an advantageous position to make minimum structural modifications to further potentiate antioxidant capacity of this medicament and formulate a new compound with greater antioxidant property as antioxidants have become an essential drug among all communities everywhere in the world. Future studies have been designed to determine the exact mechanism of action with which imipramine is able to scavenge free radicals.

CONCLUSION

Exhaustive and extensive studies by various researchers have repeatedly proved that the flavonoid quercetin present in many plants possess potent antioxidant action. Imipramine being structurally similar to quarcetin was selected to determine its antioxidant function by following known standard procedures. Imipramine turned out to be a very active antioxidant, being more potent often than ascorbic acid in two out of five important assay systems.

Based on these findings pharmaceutical industries are in a position to make necessary changes in the structure of imipramine and come to market with a new antioxidant, which is so much in demand in the present scenario of therapy.

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