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In Vitro Antioxidant Activity of Dibenz[*b,f*]azepine and its Analogues

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Abstract: Dibenz[*b,f*]azepine and its five derivatives bearing different functional groups were synthesized by known methods. The compounds thus synthesized were evaluated for antioxidant potential through different *in vitro* models such as (DPPH) free radical scavenging activity, *β*-carotene-linoleic acid model system, reducing power assay and phosphomolybdenum method. Under our experimental condition among the synthesized compounds dibenz[*b,f*]azepine (**a**) and 10-methoxy-5*H*-dibenz[*b,f*]azepine (**d**) exhibited potent antioxidant activity in concentration dependent manner in all the above four methods. Butylated hydroxyl anisole (BHA) and ascorbic acid (AA) were used as the reference antioxidant compounds. The most active compounds like dibenz[*b,f*]azepine and its methoxy group substituent have shown more promising antioxidant and radical scavengers compared to the standards like BHA and ascorbic acid. It is conceivable from the studies that the tricyclic amines, *i.e.* dibenz[*b, f*]azepine and some of its derivatives are effective in their antioxidant activity properties.

Keywords: Dibenz[*b,f*]azepine, Antioxidant activity, Radical scavenging activity, *β*-Carotene-linoleic acid, Reducing power, Phosphomolybdenum.

Introduction

Free radicals can have a noxious effect on cells, and it is believed that free radical damage is involved in the etiology of several diseases. Free radicals are being formed during normal cellular metabolism and they are known to contribute to healthy functions in human health and development when they are not excessive. Formation of free radicals is not limited to normal cellular process but also occurs upon exposure to certain chemicals (polycyclic aromatic hydrocarbon, cadmium, lead, *etc*.), radiation, cigarette smoke, and high fat diet. A balance between formation of free radicals and their detoxification is essential for normal cellule function. When such a balance is disrupted as a result of excessive generation of

damaging species or low levels of antioxidants, a cell enters a state of oxidative stress and is damaged. If the damage persists the cell will enter a state of genetic instability that can lead to chronic diseases including cancer[1]. A series of compounds that can scavenge radicals by trapping initiating and/or propagating radicals, thus called 'antioxidant'[2]. In biological systems, the definition for antioxidants has been extended to any substance that when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate[3].

Antioxidants protect against these radicals, and it is important to balance an enhanced radical production with a sufficient supply of antioxidants. Numerous natural and synthetic antioxidant compounds have been tested with success in various disease models as well as in clinics[4]. *In vivo* production of excess free radicals plays a role in several human diseases. Septic shock, triggered by bacterial endotoxins, is linked to radical-induced tissue, which leads to pulmonary and cardiac collapse and even to death[5].

In the literature some tricyclic amines and their chemical structure shows antioxidant neuroprotective activity *in vitro*[6]. Now a day, the free-radical scavenging mechanism of aromatic amines (Ar_2NHs) has been discussed from the view of chemical kinetics[7].

Dibenz[*b,f*]azepine and its derivatives has been variously reported as having antiallergic activity, specifically antihistaminic activity, spansmolytic, serotonin antagonistic, anticonvulsive, antiemetic, antiepileptic, anti-inflammatory, sedative and fungicidal action[10]. 5*H*-dibenz[*b,f*]azepine is a common basic fused tricyclic tertiary amine, related to a class of anticonvulsants which are employed in the prevention or the treatment of epileptic seizures and bipolar disorders. This nucleus has been known since 1899, when Thiele and Holzinger prepared 10,11-dihydro dibenz[*b,f*]azepine[6].The approved (chemical abstracts) numbering of the ring positions of 5H-dibenz[*b,f*]azepine is as shown in Figure 1**.**

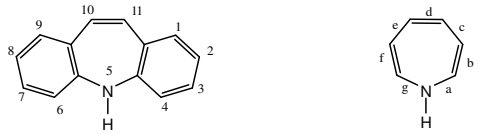


Figure 1. Numbering of the ring position of 5H-dibenz[*b,f*]azepine.

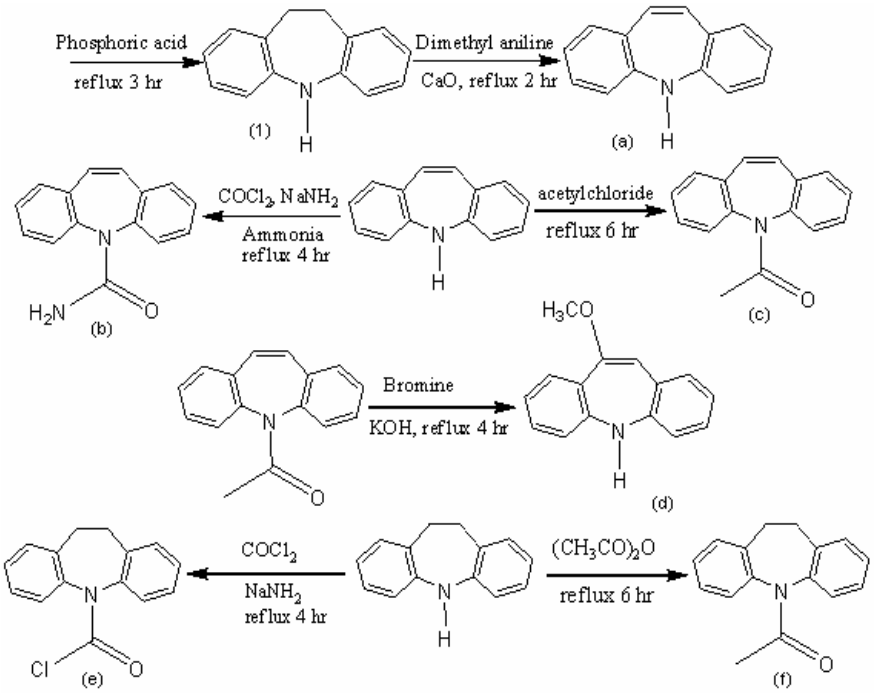
5*H*-Dibenz[*b,f*]azepine is useful for various therapeutics such as carbamazepine, oxcarbamazepine *etc*[7].

The research on antioxidants provides theoretical information for the medicinal development, and supplies some *in vitro* methods for quick-optimizing drugs, it attracts more scientific attention from bioorganic and medicinal chemists. In addition to the traditional O-H bond type antioxidant, tricyclic amines having N-H bond functions as the antioxidant have attracted much research attention because Ar_2NHs have always been the central structure in many currently used drugs. Since their structure may justify a possible intervention on free radical process, so this study has been taken to explore better the chemistry and biological activities of dibenz[*b,f*]azepine and its derivatives. Six known molecules, numbering **a** to **f** were synthesized and their structure was established by chemical and spectral analysis. The compounds were investigated for *in vitro* antioxidant potential and compared to commercially available synthetic antioxidants namely BHA and ascorbic acid.

Experimental

Melting points of the compounds were determined by the open capillary method and are uncorrected. The IR spectra were recorded on a FT-IR021 model in KBr disc and in nujol

mull. The ^{1}H NMR spectra were recorded on Jeol-60MHz and Jeol GSX 400MHz spectrophotometer using $CDCl_3$ as a solvent and TMS as an internal reference. The chemical shifts are expressed in δ (ppm) values. The Mass spectra were recorded on Hitachi RMU-61 spectrophotometer and important fragments are given with percentage of abundance in the bracket. The purity of the compounds was checked by thin layer chromatography on silica gel glass plates in hexane and ethyl acetate solvent mixture (9:1 v/v). The compounds were purified by column chromatography on silica gel (60-120 mesh) bed as adsorbent and hexane and ethyl acetate as eluent.



Reaction Scheme 1.

General procedure for the preparation of dibenz[*b,f*]azepine and its five derivatives[11].

*Dibenz[b,f]azepine (Compound **a**)*

Dibenz[*b,f*]azepine was prepared by coupling of nitro tolune in presence of basic catalyst to form bibenzyl (*o,o'* dinitroazepine). This is reduced to give 10,11-dibenz[*b,f*]azepine (1) further upon dehydrogenation yielded Dibenz[*b,f*]azepine.

*5H-Dibenz[b,f]azepine-5-carboxamide (Compound **b**)*

Dibenz[*b,f*]azepine (1.93 g, 10 mM) was refluxed in the presence of $COCl_2$ with strong base ($NaNH_3$) to get chloro carbonyl dibenz[*b,f*]azepine (10 mM, 0.253 g), which upon further reflux with concentrated ammonia (25 mL) to give product.

*1-DIbenz[b,f]azepine 5yl) ethanone (Compound **c**)*

5H-dibenz[*b,f*]azepine 5yl) ethanone was prepared by refluxing 5*H*-dibenz[*b,f*]azepine (1.93 g, 10 mM) in acetic anhydride (25 mL) for 6h.

*10-Methoxy-5H-dibenz[b,f azepine (Compound **d**)*

10-Methoxy-5*H*-dibenz[*b,f*]azepine was prepared by brominating N-acetyl-5H-dibenz[*b,f*] azepine (2.35 g, 10 mM) using bromine (3.2 g, 20 mM) in dichloromethane (25 mL) to obtain dibromo derivative, which was further refluxed with KOH (1.12 g, 20 mM) in CH_3OH (25 mL) to obtain the product.

*5-Chloro carbonyl-10,11-dihydro-5H-dibenz[b,f]azepine (Compound **e**)*

5-Chlorocarbonyl-10,11-dihydro-5*H*-dibenz[*b,f*]azepine obtained by refluxing dihydro-5*H*-dibenz [*b,f*]azepine (1.95g, 10 mM) with concentrated triphosgene (25 mL) in presence of *di*base ($NaNH_2$).

*1-(10,11-Dihydrobenz[b,f azepine 5yl) ethanone (Compound **f**)*

1-(10,11-Dihydrobenz[*b,f*]azepine 5yl) ethanone was prepared by refluxing 10,11-dihydro-5*H*-dibenz[*b,f*]azepine (1.95 g, 10 mM) in acetic anhydride (25 mL) for 6h.

Antioxidant studies

In the present study, four commonly used antioxidant evaluation methods such as DPPH radical scavenging activity, *β*-carotene -linoleic acid model system, reducing power assay and phosphomolybdenum method were chosen to determine the antioxidant potential of the six compounds. The synthesized compounds were dissolved in distilled ethanol (50 mL) to prepare 1000 µM solution. Solutions of different concentrations (10 µM, 50 µM, 100 µM, 200 µM and 500 µM) were prepared by serial dilution and the free radical scavenging activity and antioxidant activity evaluation were studied.

DPPH radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging effect was carried out according to the method first employed by Blois[12]. Compounds of different concentrations were prepared in distilled ethanol, 1 mL of each compound solutions having different concentrations (10 µM, 50 µM, 100 µM, 200 µM and 500 µM) were taken in different test tubes; 4 mL of a 0.1 mM ethanol solution of DPPH was added and shaken vigorously. The tubes were then incubated in the dark room at RT for 20 min. A DPPH blank was prepared without compound, and ethanol was used for the baseline correction. Changes (decrease) in the absorbance at 517 nm were measured using a UV-visible spectrophotometer and the remaining DPPH was calculated. The percent decrease in the absorbance was recorded for each concentration, and percent quenching of DPPH was calculated on the basis of the observed decreased in absorbance of the radical. The radical scavenging activities were expressed as the inhibition percentage and were calculated using the formula:

$$\text{Radical scavenging activity } (\%) = [(A_0 - A_1 / A_0 \times 100]$$

Where A_0 was the absorbance of the control (blank, without compound) and A_1 was the absorbance of the compound. The radical scavenging activity of BHA and ascorbic acid was also measured and compared with that of the different synthesized compound. For all the compounds and standards half inhibition concentration (IC_{50}) was calculated graphically using a linear regression algorithm.

Antioxidant activity by β- carotene – linoleic acid assay

Each compound at the final concentration of 50 µM/mL and 100 µM/mL were incorporated into *β*-carotene-linoleic acid model system independently and the activity was monitored spectrophotometrically at 470 nm[13].

Preparation of the suspension

The substrate suspension was prepared by addition of *β*-carotene (4 mg dissolved in 5 mL

chloroform) into a covered round bottomed flask containing Tween- 40 (600 mg) followed by the addition of linoleic acid (60 µL). The chloroform was removed completely under vacuum using rotavapour at 40^0C. The resulting solution was diluted with triple distilled water (30 mL) and the emulsion was mixed well and the diluted with oxygenated water (120 mL).The aliquots (4 mL) was transferred to different stopper test tubes containing compound (50 and 100 µM/mL) in distilled alcohol. Control was prepared with distilled alcohol (1 mL) and emulsion (4 mL). BHA and ascorbic acid solution as internal standards of the same concentration were also analyzed for comparison. Zero adjustment was done using distilled water. Absorbance of the samples was measured at a wavelength of 470 nm, immediately (t=0), and subsequently after every 30 min for 3hr (t=180). The tubes were placed in a water bath at 50^0C between the readings. Antioxidant activities (AA) of each compound were evaluated in triplicates in terms of photooxidation of β-carotene using the following formula:

$$AA= 100\ [1- (Ao – A_t / Ao^o - Ao^t)]$$

Where, AA = Antioxidant Activity.
A_o = Initial absorbance of the sample.
A_t = Absorbance of the sample after time‘t’.
A_o^o = Initial absorbance of the control.
A_o^t = Absorbance of control after time‘t’.

Reducing power assay (Iron reducing activity)

The reducing power of synthesized compounds was determined according to the method of Oyaizu[14]. The compounds having 50 µM and 100 µM were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferric cyanide, and then incubated at 50^0C for 20 min. To this mixture 2.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 3000 rpm for 20 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride and the absorbance was taken at 700nm. Increased absorbance of the reaction mixture indicates an increased reducing power

Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity was evaluated by the method of Prieto *et al*[15]. An aliquot of 0.1 mL of compound solutions (50 µM, 100 µM) was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). In case of blank 0.1 mL of methanol was used in place of compound. The tubes were capped and incubated in a boiling water bath at 95^0C for 90 min. After the samples had cooled to RT, the absorbance of the aqueous solution of each was measured at 695 nm against blank in spectrophotometer. For compound of unknown composition, antioxidant capacity was expressed as equivalent of ascorbic acid (µM/mg of compound).

Statistical analysis

Tests were carried out in triplicates. The amount of compound needed to inhibit free radicals concentration by 50% (IC_{50}) was graphically estimated using a linear regression algorithms.

Results and Discussion

DPPH radical scavenging activity

The scavenging effects of all of the synthesized compounds on the DPPH free radical were evaluated. Now-a-days, antioxidants that exhibit DPPH radical scavenging activity are increasingly receiving attention. The model of scavenging of the stable DPPH radical is extensively used to evaluate radical scavenging activities in less time than other methods.Compound reacts with DPPH, which is a nitrogen centered radical with a characteristic

absorption at 517 nm, and convert it to stable diamagnetic molecule 1,1-diphenyl-picryl hydrazine, due to its hydrogen donating ability at a very rapid rate[16]. When this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electrons taken up. Such a change in the absorbance produced in this reaction has been widely applied to test the capacity of numerous molecules to act as free radical scavengers[18]. The six synthesized compounds scavenged DPPH radical significantly in a concentration-dependent manner.

The radical scavenging activities of the synthesized compounds are summarized in Table 1. Compounds **a** and **d** showed appreciable radical scavenging activity. The presence of N-H group which can donate hydrogen atom in compound a moiety may contribute to the radical scavenging activity. The N-H group does have the radical scavenging property as the N protected compound[19]. The presence of the electron donating -OCH_3 group at the 11th position of the seven member ring with N-H group may also contribute for better activity than dibenz[*b,f*]azepine whereas, the presence of carbonyl group in the other compounds **b**, **c**, **e**, **f** may hinder the scavenging ability and shows negligible scavenging activity over DPPH. The presence of methoxy group in the seven member ring may enhance the stability of the nitrogen centered radical due to electron conjugation effect. 50% Inhibition concentration (IC_{50}) for the synthesized compounds and the standards were calculated from the Table compound **a** and **d** displayed better activity than the standards.

Table 1. Radical scavenging activity (%) of dibenz[*b,f*]azepine and its derivatives. each value represents means ± SD (n=3)

Compound name	RSA, % Concentration, µM					IC_{50} µM/mL
	10	50	100	200	500	
a	52.86±0.64	71.51±0.92	80.76±1.02	85.44±1.10	91.94±0.32	4.46
b	0.97±0.44	1.72±1.22	2.25±0.54	7.38±0.98	9.37±0.33	270.10
c	0.11±1.29	0.35±1.20	0.54±1.22	1.60±1.34	4.38±0.13	187.26
d	69.60±0.18	78.25±0.34	86.03±0.82	92.22±1.45	95.71±0.11	2.10
e	4.19±1.23	6.38±1.22	6.62±1.32	12.50±2.34	13.89±1.37	197.32
f	0.21±1.78	0.35±1.39	0.54±1.44	1.79±1.19	4.38±1.87	187.26
AA	45.64±0.67	62.61±0.39	75.66±0.98	81.55±0.21	84.39±0.10	4.94
BHA	45.81±0.99	68.23±1.00	73.76±0.22	77.30±0.11	83.53±0.12	5.26

β-Carotene- linoleic acid model system

This method is based on the determination of the coupled oxidation of carotene and linoleic acid. The basic principle involved is that linoleic acid, unsaturated fatty acids get oxidized by reactive oxygen species (ROS) produced by oxygenated water. The products formed initiate the *β*-carotene oxidation, which leads to discoloration. Bleaching of *β*-carotene a free radical mediated phenomenon resulting from hyperoxides formed from linoliec acid. The six synthesized compounds scavenged DPPH radical significantly in a concentration-dependent manner. *β*-Carotene looses its double bonds by oxidation which results in lose of its characteristic orange colour monitored spectrophotometrically at 470 nm. Bleaching of *β*-carotene with respect to time of synthesized compounds at different concentrations are represented in Figure 1. The Figure indicates that due to the oxidation of linoleic acid which eventually attacks the highly unsaturated *β*-Carotene molecule undergo rapid discoloration, compound **b**, **c**, **e**, **f** looses its chromophore when it allowed for oxidation during different time interval (0-180 min). Initially the absorbance of the compounds was more and decreased drastically with in the time allowed for oxidation, but in case of compound **a** and **d** there is negligible decrease in the absorbance was observed.

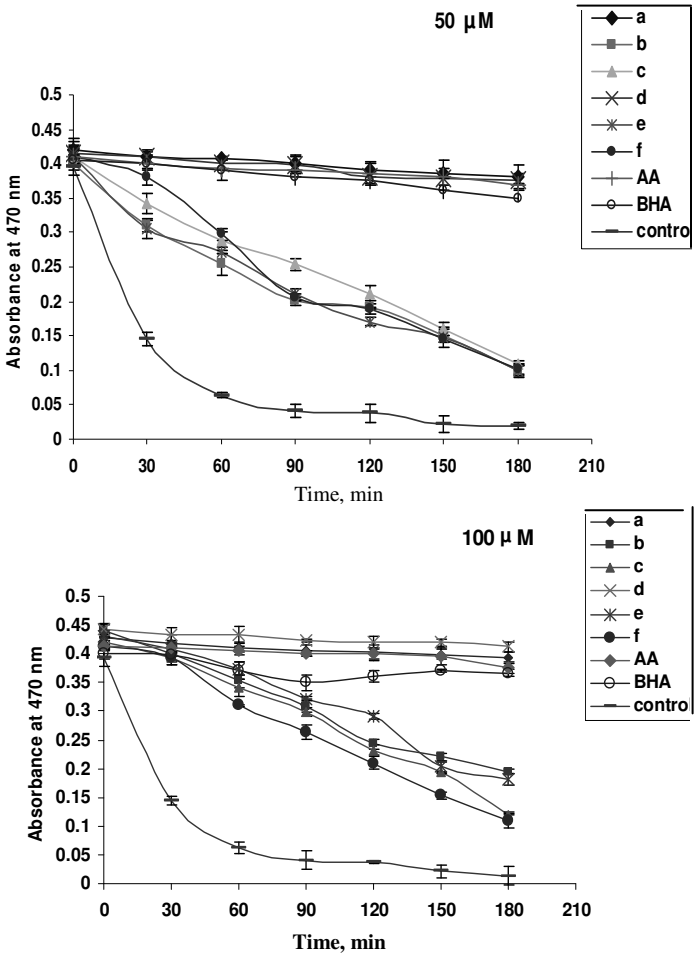


Figure 1. Absorbance change of *β*-carotene at 470 nm in the presence of compounds **a** to **f**, control and positive control (BHA, AA). Each value represents means ± SD (n=3)

The control (no additive) are decolourised within 150 min, indicating that rapid oxidation occurred. The addition of compounds a to f and positive controls (BHA and AA) at different concentrations enhanced the bleaching time of *β*-Carotene.

The presence of antioxidant compounds a and d, binds the extent of *β*-Carotene bleaching by neutralizing the linoleate free radical; hence extent of decrease in discoloration indicates higher antioxidant activity.

The antioxidant activity for dibenz[*b,f*]azepine and its five derivatives was calculated and the percentage activities are as shown in Figure 2.The Figure indicates that there is significant difference in the activity (%) at different concentrations. It was observed that the antioxidant activities (%) of all the compounds increase with increase in concentrations.

This effect of concentration on antioxidant activity becomes more pronounced as the time allowed for oxidation increases. The antioxidant activities of compounds were compared to the standards (BHA, ascorbic acid) and the compounds **b**, **c**, **e**, **f** possess less antioxidant activity but compound **a** and **d** showed promising antioxidants. Also the

antioxidant activity of **a** and **d** remained stable even with the time increase allowed for oxidation, where as **b**, **c**, **e**, **f** decreased drastically as the time of oxidation increased. This was particularly noticeable at lower concentration (50 µM). This is because compound **a** contains free amino group (N-H bond) which can quenches the radical. The introducing of electron-with drawing group (-OCH_3) on the seven membered ring of the compound a leads considerable increase in the scavenging activity. Where as in the case of compound **b**, **c**, **e**, **f** the presence of carbonyl group may hinder the antioxidant capacity. Hence in this assay compound **a** and **d** increases and stabilizes the antioxidant activity compared to other compounds. The activity was also compared to the commercial synthetic standards like BHA, ascorbic acid. From the comparison compound **a** and **d** showed equal and even more DPPH radical scavenging activity.

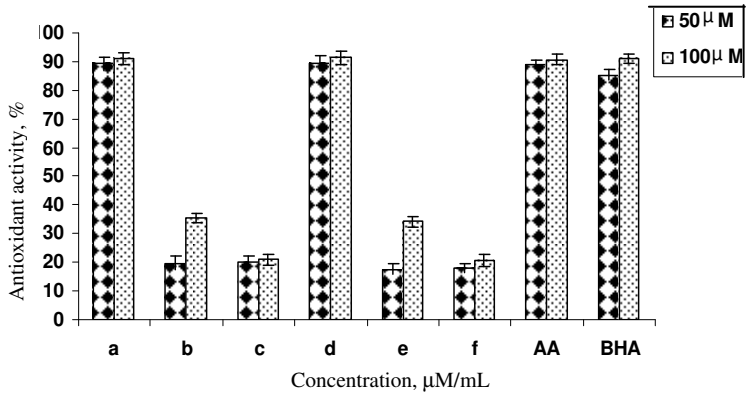


Figure 2. Antioxidant activity (%) of the dibenz[*b,f*]azepine and its derivatives at 50 µM and 100 µM concentration in *β*-Carotene-Linoleic acid system. Values are means of triplicate determination ± standard deviation.

Reducing power assay

Reducing power of dibenz[*b,f*]azepine and its five derivatives at different concentration (25 µM, 50 µM, 100 µM and 200 µM) was determined. In this assay, depending on the reducing power of antioxidant compounds the test solutions changes into various shades of green and blue colors. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reduction of ferricyanide (Fe^{3+}) complex to the ferrous (Fe^{2+}) form can be monitored by measuring the formation of Perl's Prussian blue at 700 nm which occurs in the presence of reductants (antioxidant compounds). Reducing power of the dibenz[*b,f*]azepine, its derivatives and standards (BHA and ascorbic acid) using the potassium ferricyanide reduction method were depicted in Figure 3. Compound **a**, **d** possess good reducing power ability compared to the other compounds. The Figure indicates that the compound a having free N-H group reduces free radicals but compound d having methoxy group in addition to N-H group reduces considerably more than compound **a**. These two compounds may react with free radicals to convert them to more stable products and terminate radical chain reaction. The presence of carbonyl group in the compound (**b**, **c**, **e** and **f**) may hinder the reduction of Fe^{3+} to Fe^{2+} and shows the negligible reducing ability. In general, the reducing power observed in the present study was in the following order compound d > compound a > BHA > ascorbic acid > compound **b**, **c** and **e**. The data presented here indicate that the marked reducing power of compound **a** and **d** seems to be the result of their antioxidant activity.

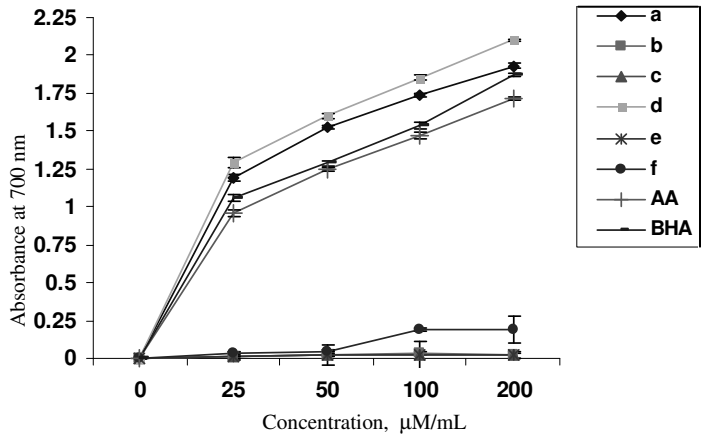


Figure 3. Antioxidant activity of the dibenz[*b,f*]azepine and its derivatives at different concentrations using reducing power assay. Each value represents means ± SD (n=3). High absorbance at 700 nm indicates high reducing power

The reducing power of the internal standards (BHA, ascorbic acid) was also carried out and comparative study over the synthesized compounds was done. Compound **a** and **d** shows promising activity than the internal standards.

Evaluation of antioxidant capacity by phosphomolybdenum method

The antioxidant activity for the synthesized compounds was evaluated by using phosphomolybdate method. It determines the total antioxidant capacity. This assay is based on the reduction of Mo(VI) to Mo(V) in presence of the antioxidant compounds and the subsequent formation of a green phosphate/Mo(V) complex at acidic p^H, which is measured at 695 nm.The antioxidant capacity of the compounds was determined for 25 µM, 50 µM and 100 µM concentrations. The antioxidant capacities of the compounds determined by phosphomolybdate method was expressed as µM of ascorbic acid equivalent/mg and showed in the Figure 4

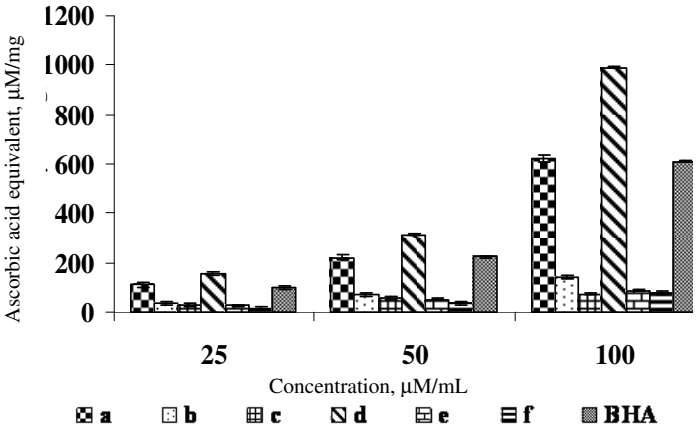


Figure 4. Antioxidant capacities of dibenz[b,f]azepine and its derivatives by phosphomolybdate method. Each value represents means ± SD (n=3)

From the Figure compound a showed good antioxidant capacity whereas compounds **b**, **c**, **e** and **f** having carbonyl group may hinder the reducing ability and shows less antioxidant capacity. Compound **d** reduces Mo(VI) to Mo(V) in better way due to the presence of -OCH_3 group in addition to N-H group shows the better activity than compound a. The antioxidant activity of the compounds were compared to standards (BHA and ascorbic acid) and found that compound a and d possess better phosphomolybdate activity than the standards.

Conclusions

Dibenz[*b*,*f*]azepine and its five analogues were successfully synthesized from the known methods and the antioxidant activity for the synthesized compounds was evaluated. The results of the present experiment shows that compound **a** and **d** showed more promising antioxidant activity against DPPH radical scavenging activity, reducing power assay, *β*-carotene-linoleic acid model system and phosphomolybdate method. The most active compounds like dibenz[*b*,*f*]azepine and its derivative (10-Methoxy-5*H*-dibenz[*b*,*f*]azepine) have shown better antioxidant and radical scavengers compared to the standards like BHA and ascorbic acid. It is conceivable from the studies that the tricyclic amines *i.e.,* dibenz[*b*,*f*] azepine and some of its analogues are effective in their antioxidant activity properties. This will provides the theoretical information for the medicinal development, and supplies some *in vitro* methods for quick-optimizing drugs, which can be useful for the potential source of synthetic antioxidant.

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