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Evaluation of In Vitro Antioxidant Activity of
5H-dibenz[b,f]azepine and Its Analogues

Vijay Kumar Honnaiah[1], Ranga Rao Ambati[2], Varakumar Sadineni[3] and Nagaraja Naik[1]*

[1]Department of Studies in Chemistry, University of Mysore, Manasagangotri
Mysore – 570 006, Karnataka, India
[2]Department of Oils and Fats, V. R. S. & Y. R. N. College,
Acharya Nagarjuna University, Chirala – 523 157, Andhra Pradesh, India
[3]Department of Biochemistry, Sri Venkateswara University,
Tirupati – 517 502, Andhra Pradesh, India

*Corresponding author: drnaik_chem@yahoo.co.in

Abstract: *Synthesis of 5H-dibenz[b,f]azepine and its derivatives bearing different functional groups was performed. The compounds thus synthesised were evaluated for their antioxidant activities by following two well established assays: inhibitory activity on human low density lipoprotein (LDL) oxidation and lipid peroxidation activity in an egg liposome model system. Among the compounds, (a) and (d) significantly inhibited human LDL oxidation and liposome peroxidation, whereas compounds (b), (c), (e) and (f) showed less activity. Butylated hydroxy anisole (BHA) and ascorbic acid (AA) were used as reference antioxidant compounds. Comparative studies with the synthesised compounds were also performed.*

Keywords: 5H-dibenz[b,f]azepine, LDL oxidation, lipid peroxidation, antioxidant activity

1.   INTRODUCTION

Free radicals and active oxygen species have been correlated with cardiovascular and inflammatory diseases and even with a role in cancer and ageing.[1,2] Efforts to counteract the damage caused by these species are gaining acceptance as a basis for novel therapeutic approaches, and the field of preventive medicine is experiencing an upsurge of interest in medically useful antioxidants.[3,4] Recent evidence[5] suggests that free radicals, which are generated in many bioorganic redox processes, may induce oxidative damage in various components of the body (e.g., lipids, proteins and nucleic acids) and may also be involved in processes leading to the formation of mutations. Furthermore, radical reactions play a significant role in the development of life-limiting chronic diseases such as cancer, diabetes, arteriosclerosis and others.[6] It has been suggested that oxidative modification of low-density lipoproteins (LDLs) may play a role in the development of atherosclerosis.[7] The oxidative modification

depends on a common initiating step, the peroxidation of polyunsaturated fatty acid components of LDLs.[8] Such modification of LDLs can be inhibited by antioxidants.[9,10]

In the literature some tricyclic amines and their chemical structures show antioxidant neuriprotective activity in vitro.[11] Nowadays, the antioxidant mechanism of aromatic amines (Ar$_2$NHs) has been discussed from the view of chemical kinetics.[12] 5H-dibenz[b,f]azepine i.e., iminostilbene (Fig. 1) is a common basic fused tricyclic amine. It is used as an intermediate for the synthesis of the registered anticonvulsant drug oxcarbazepine,[13] the structure of which has recently been reported.[14] Dibenz[b,f]azepine and its derivatives have been variously reported as having antiallergic activity, specifically antihistaminic, spasmolytic, serotonin antagonistic, anticonvulsive, antiemetic, antiepileptic, anti-inflammatory, sedative and fungicidal action.[15]
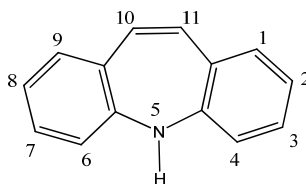

Figure 1: Structure of 5H-dibenz[b,f]azepine.

The research on free radicals provides theoretical information for the medicinal development and supplies some in vitro methods for quickly optimising drugs; it is attracting increased scientific attention from bioorganic and medicinal chemists. Generally, phenolic compounds are found to have antioxidant and radical scavenging activity, and they also inhibit LDL oxidation.[16,17] In addition to the traditional O–H bond type antioxidants, tricyclic amines, having N–H bond functions as the antioxidant, have attracted much research attention because Ar$_2$NHs are the central structure in many currently used drugs.[18] Recently, we have reported the antioxidant properties of 5H-dibenz[b,f]azepine and some of its analogues, and it was possible to establish some structure-activity relationships based on the different substitutes and the positions.[19]

As their structures may justify a possible intervention in the free radical process, therefore this study has been taken to explore better the chemistry and antioxidant properties of 5H-dibenz[b,f]azepine and its derivatives. Six molecules (a–f) were synthesised, and their structures were established by chemical and spectral analysis. The synthesised compounds were investigated for in vitro antioxidant potential, and a comparative study was done on commercially

available synthetic antioxidants, namely butylated hydroxy anisole (BHA) and ascorbic acid (AA).

2.   EXPERIMENTAL

2.1   Protocols

The following reagents were obtained from Sigma Chemicals Co. (St. Louis, MO, USA): 1,1,3,3 tetra methoxy propane and malanaldehyde. Copper sulphate, sodium dihydrogen ortho phosphate disodium ortho phosphate, TBA, TCA, NaCl, ferric chloride, L-ascorbic acid, HCl and NaOH were of analytical grade and obtained from Merck, Mumbai, India. Melting points of the compounds were determined by the open capillary method and are uncorrected. The IR spectra were recorded on a FT-IR 021 model (Perkin Elmer, Massachusetts, USA) in a KBr disc and in nujol mull. The ^{1}H NMR spectra were recorded on a Jeol-60 MHz and Jeol GSX 400 MHz spectrophotometer (Joel Ltd., Tokyo, Japan) using CDCl$_3$ as a solvent and tetramethylsilane (TMS) as an internal reference. The chemical shifts are expressed in δ (ppm) values. The mass spectra were recorded on a Hitachi RMU-61 spectrophotometer (Hitachi Seisakusho Co. Ltd., Tokyo, Japan), and important fragments are given with the percentage of abundance in brackets. The purity of the compounds was checked by thin layer chromatography on silica gel glass plates in a hexane and ethyl acetate solvent mixture (9:1 v/v). The compounds were purified by column chromatography on a silica gel (60–120 mesh) bed as adsorbent and hexane and ethyl acetate as eluent (9:2 v/v).

2.1.1   Procedure for the preparation of 5H-dibenz[b,f]azepine (Compound a)

5H-dibenz[b,f]azepine (a) was prepared by the coupling of *o*-nitro toluene (2 mM) in methanol in the presence of the catalyst ethyl formate and KOH (1 mM) in methanol by refluxing for 4 hr to form bibenzyl (*o,o'*-dinitroazepine). This was reduced to give 10,11-dihydro-5H-dibenz[b,f]azepine (1) upon refluxing with phosphoric acid, a cyclisation agent, for 3 hr and dehydrogenation with CaO in dimethyl aniline solution upon refluxing for 2 hr to obtain 5H-dibenz[b,f]azepine.

Orange yellow solid, yield 82%, m.p. 197°C–201°C. IR (KBr)v_{max} (cm^{-1}): 3360.0 (N–H), 3046.3 (Ar–H). ^{1}H NMR (δ, CDCl$_3$): 3.3 (s, 1H, N–H), 6.7–8.1 (m, 8H, Ar–H), 7.0 (m, 2H, 7 membered Ar–H). Mass (%): M$^+$ 193.16 (90), 195 (5), 196 (11). Anal. Calc. for C$_{14}$H$_{11}$N: C, 87.01; H, 5.74; N, 7.25. Found: C, 87.00; H, 5.77; N, 7.26.

2.1.2   Procedure for the preparation of 5H-dibenz[b,f]azepine-5-carboxamide (Compound b)

5H-dibenz[b,f]azepine (1.93 g, 10 mM) was refluxed in the presence of $COCl_2$ with a strong base ($NaNH_2$) for 4 hr to obtain chloro carbonyl dibenz[b,f]azepine (0.253 g, 10 mM), which upon further reflux with concentrated ammonia (25 ml) yielded 5H-dibenz[b,f]azepine-5-carboxamide.

White solid, yield 81%, m.p. 190°C–193°C. IR (KBr)v_{max} (cm^{-1}): 3421.0–3465.4 (NH_2), 3163.4 (Ar–H), 1671 (C=O). ^{1}H NMR (δ, $CDCl_3$): 6.9 (s, 2H, NH_2), 7.3–7.5 (m, 8H, Ar–H), 7.0 (m, 2H, 7 membered Ar–H). Mass (%): M$^+$ 236.15 (88), 238 (7), 269 (11), 239 (1). Anal. Calc. for $C_{15}H_{12}N_2O$: C, 76.25; H, 5.12; N, 11.86; O, 6.77. Found: C, 76.26; H, 5.13; N, 11.88; O, 6.74.

2.1.3   Procedure for the preparation of 1-5H-dibenz[b,f]azepine-5yl)ethanone (Compound c)

1-5H-dibenz[b,f]azepine-5yl)ethanone was prepared by refluxing 5H-dibenz[b,f]azepine (1.93 g, 10 mM) in acetic anhydride (25 ml) for 6 hr.

Brown solid, yield 85%, m.p. 159°C–162°C. IR (KBr)v_{max} (cm^{-1}): 3069.0 (Ar–H), 1668.9 (C=O). ^{1}H NMR (δ, $CDCl_3$): 7.2–7.5 (m, 8H, Ar–H), 7.0 (d, 2H, 7 membered Ar–H), 2.0 (s, 3H, CH_3). Mass (%): M$^+$ 235.18 (91), 238 (10), 239 (11). Anal. Calc. for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95; O, 6.80. Found: C, 81.69; H, 5.55; N, 5.98; O, 6.81.

2.1.4   Procedure for the preparation of 10-methoxy-5H-dibenz[b,f]azepine (Compound d)

10-methoxy-5H-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. Furthermore, to the above solution, KOH (1.12 g, 20 mM) in CH_3OH (25 ml) was added and refluxed for 4 hr to obtain the product.

Yellow solid, yield 87%, m.p. 181°C–183°C. IR (KBr)v_{max} (cm^{-1}): 3416.0–3469.1 (NH_2), 3163.4 (Ar–H), 1690 (C=O). ^{1}H NMR (δ, $CDCl_3$): 3.3 (s, 1H, N–H), 6.8–7.9 (m, 8H, Ar–H), 6.9 (m, H, 7 membered Ar–H), 3.8 (s, 3H, OCH_3). Mass (%): M$^+$ 223.15 (88), 225 (7), 227 (11). 229 (1). Anal. Calc. for $C_{15}H_{13}NO$: C, 80.69; H, 5.87; N, 6.27; O, 7.17. Found: C, 80.68; H, 5.88; N, 6.25; O, 7.18.

2.1.5 Procedure for the synthesis of 5-chlorocarbonyl-10-11-dihydro-5H-dibenz[b,f]azepine (Compound e)

5-chlorocarbonyl-10-11-dihydro-5H-dibenz[b,f]azepine was obtained by reacting 10,11-dihydro-5H-dibenz[b,f]azepine (1.95 g, 10 mM) with $COCl_2$ (25 ml) in the presence of triethyl amine as base at RT for 8 hr.

White solid, yield 91%, m.p. 149°C–151°C. IR (KBr)v_{max} (cm^{-1}): 3163.4 (Ar–H), 1683 (C=O). ^{1}H NMR (δ, CDCl$_3$): 7.2–7.6 (m, 8H, Ar–H), 3.1 (s, 4H, 7 membered ring). Mass (%): M$^+$ 257.47 (82), 259 (10), 260 (1), 261 (1). Anal. Calc. for $C_{15}H_{12}NOCl$: C, 69.91; H, 4.69; N, 5.43; Cl, 13.76. Found: C, 69.90; H, 4.67; N, 5.44; Cl, 13.77.

2.1.6 Procedure for the synthesis of 1-(10,11-dihydro-5H-dibenz[b,f]azepin-5-yl)ethanone (Compound f)

1-(10,11-dihydro-5H-dibenz[b,f]azepin-5-yl)ethanone was prepared by reacting 10,11-dihydro-5H-dibenz[b,f]azepine (1.95 g, 10 mM) in acetylchloride (25 ml) for 6 hr at RT.

White solid, yield 88%, m.p. 153°C–156°C. IR (KBr)v_{max} (cm^{-1}): 3163.4 (Ar–H), 1690 (C=O). ^{1}H NMR (δ, CDCl$_3$): 7.3–7.6 (m, 8H, Ar–H), 3.0 (s, 4H, 7 membered Ar–H) 3163.4 (Ar–H), 1.9 (s, 3H, CH3). Mass (%): M$^+$ 237.17 (79), 239 (7), 240 (11), 242 (1). Anal. Calc. for $C_{16}H_{15}NO$: C, 80.98; H, 6.37; N, 5.90; O, 6.74. Found: C, 80.96; H, 6.37; N, 5.92; O, 6.73.

2.2 Chemistry

In the present work, 5H-dibenz[b,f]azepine and some of its derivatives were synthesised according to the published literature[13] with slight changes in the chemical reagents and conditions. The reaction sequences are outlined in schemes 1–3. In scheme 3, to obtain compound (e), the reaction was carried out by using a weak base (triethyl amine) with $COCl_2$ at room temperature (RT) instead of triphosgene in the presence of $NaNH_2$ as a strong base in the reflux condition, and compound (f) was obtained by using acid chloride, i.e., acetyl chloride, at RT instead of using acetic anhydride in the presence of $NaNH_2$, a strong base in the reflux condition.

2.3 Pharmacology

In the present study, the synthesised compounds (a–f) were evaluated for their inhibitory activity on human LDL oxidation and antilipid peroxidation activity in a liposome model system. The compounds were dissolved in distilled

ethanol (50 ml) to prepare 1000 µM solutions. Solutions of different concentrations (5, 10, 15, 25, 50 and 100 µM) were prepared by serial dilution.

2.3.1   Inhibitory activity of lipid peroxidation in egg liposome model

The lipid peroxidation-inhibitory activity of the 5H-dibenz[b,f]azepine and its analogues in a liposome model system was determined according to the published method. [20]

Egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in an ultrasonic homogeniser (Son plus HD 2200, Bandelin Company, Berlin, Germany). Compounds of different concentrations (5, 10 and 15 µM/ml) were added to 1 ml of the liposome mixture and to the control (without test samples). Lipid peroxidation was induced by adding 10 µl of $FeCl_3$ (400 mM) and 10 µl of L-ascorbic acid (200 mM). After incubation at 37^oC for 1 hr, the reaction was terminated by adding 2 ml of 0.25 N HCl containing 150 mg/ml trichloroacetic acid (TCA) and 3.75 mg/ml of thiobarbutaric acid (TBA). The reaction mixture was subsequently boiled for 15 min, cooled to RT and centrifuged at 1500 rpm for 15 min, and the absorbance (optical density, OD) of the supernatant was read at 532 nm with a spectrophotometer. An identical experiment was performed in the absence of the compound to determine the amount of lipid peroxidation obtained in the presence of inducing agents as a control experiment. The percentage of antilipid peroxidative activity (% ALP) was calculated using the following equation:

$$ALP\ (\%) = [1 - (\text{sample OD/blank OD})] \times 100$$

2.3.2   Inhibition of human LDL oxidation

Fresh blood was obtained from fasting adult human volunteers, and plasma was immediately separated by centrifugation at 1500 rpm for 10 min at 4^oC. LDL [0.1 mg LDL protein/ml] was isolated from freshly separated plasma by preparative ultra centrifugation using a Beckman L8–55 ultra centrifuge (United Biomedical Sales & Service Corp., New York). The LDL was prepared from the plasma[21] using a differential ultra centrifugation. Protein was estimated in compounds by using the method as in Lowry *et al.*[22] The isolated LDL was extensively dialysed against phosphate buffered saline (PBS) at pH 7.4 sterilised by filtration (0.2-µm Millipore membrane system, USA) and stored at 4^oC under nitrogen. Plasma was separated from blood drawn from human volunteers and stored at 4^oC until used. Compounds with various concentrations (5, 10 and 15 µM) were taken in test tubes, and 40 µl of copper sulphate (2 mM) was added; the volume was increased to 1.5 ml with phosphate buffer (50 mM, pH 7.4). The test tube without compound and with copper sulphate served as a negative

control, and another test tube with compound and without copper sulphate served as a positive control. All the tubes were incubated at 37°C for 45 min. A 1 ml aliquot was drawn at 2, 4 and 6 hr intervals from each test tube, and 0.25 ml of TBA (1% in 50 mM NaOH) and 0.25 ml of TCA (2.8%) were added. The tubes were again incubated at 95°C for 45 min. Furthermore, the tubes were cooled to RT and centrifuged at 2500 rpm for 15 min. A pink chromogen (malondialdehyde, MDA) was extracted by centrifugation at 200 rpm for 10 min, and the absorbance was recorded at 532 nm using a spectrophotometer against an appropriate blank. Data were expressed in terms of MDA equivalent, estimated by comparison with standard graph drawn for 1,1,3,3-tetramethoxy-propane (which was used as a standard), which gave the amount of oxidation. The results were expressed as protection per unit of protein concentration [0.1 mg LDL protein/ml]. Using the amount of MDA, the percentage protection was calculated using the formula:

(oxidation in control – oxidation in experimental/oxidation in control) × 100.

3.   RESULTS AND DISCUSSION

In the present work, 5H-dibenz[b,f]azepine and its analogues were synthesised. Schemes 1–3 illustrate the preparation of the target molecules. As a starting material nitro toluene was used to produce 10,11-dihydro-5H-dibenz[b,f]azepine (1) and 5H-dibenz[b,f]azepine (a) (Scheme 1). Furthermore, these two molecules were used for the preparation of the derivatives (b–f) (Schemes 2 and 3). The structures of the compounds were elucidated by IR, ^{1}H NMR, mass spectroscopy and elemental analysis. The IR spectra of compounds (e) and (f) showed the absent of the N–H absorption band at 3400 cm^{-1} and the presence of the C=O stretching band at 1600 cm^{-1}, whereas in compounds (a) and (d), the presence of N–H stretching and the absence of C=O stretching were observed. In the ^{1}H NMR spectra, compounds (a) and (d) showed the N–H proton as a singlet at about 3.3 ppm, but it was not observed in compounds (b), (c), (e) and (f). All the other aromatic protons were observed at the expected regions in all the synthesised compounds. The mass spectra of compounds showed the M^{+} peak, in agreement with their molecular formula.
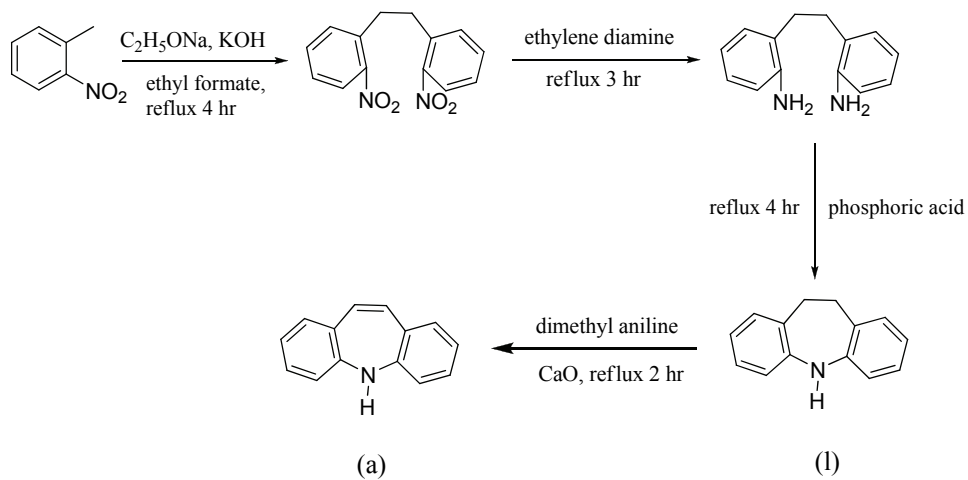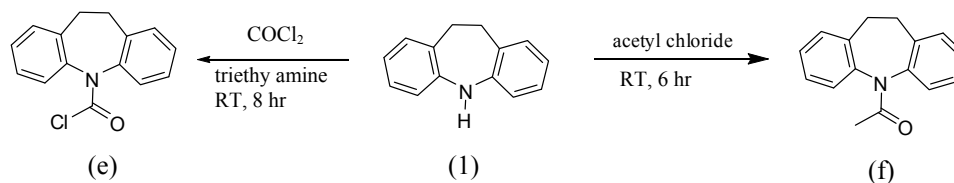
Scheme1: Protocol for the synthesis of compound (a).

Note: (1) represents 10,11-dihydro-5H-dibenz[b,f]azepine
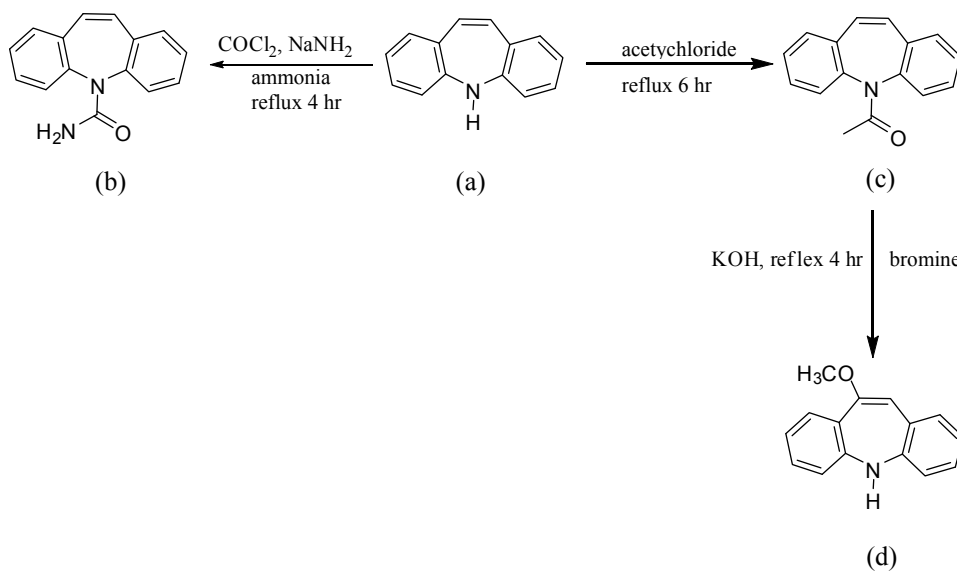
Scheme 2: Protocol for the synthesis of compounds (b), (c) and (d).

Scheme 3: Protocol for the synthesis of compounds (e) and (f).

Note: (1) represents 10,11-dihydro-5H-dibenz[b,f]azepine

In biological systems, MDA is a highly reactive species and takes part in the cross-linking of DNA with proteins and also damages liver cells.[23] Lipid peroxidation has been broadly defined as the antioxidative deterioration of polyunsaturated lipids. The initiation of a peroxidation sequence in a membrane or unsaturated fatty acid is due to extraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to be stabilised by a molecular rearrangement to produce a conjugate diene, which then easily reacts with an oxygen molecule to give a peroxy radical.[24] Peroxy radicals can extract a hydrogen atom from another molecule, or they can extract a hydrogen atom to give a lipid hydroperoxide, R–OOH. A probable alternative fate of the peroxy radical is to form a cyclic peroxide; these cyclic peroxidase, lipid peroxides and cyclic endoperoxides fragment to aldehydes including MDA and polymerisation products. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process in egg lecithin.

Lipid peroxidation is a free radical meditated propagation of oxidative damage to polyunsaturated fatty acids involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants. TBA reacts with MDA to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. The lipid peroxidation activity of the 5H-dibenz[b,f]azepine and its derivatives in the liposome system, induced by $FeCl_3$ plus AA, is represented in Figure 2. Compound (a) and (d) showed promising ALP activity like AA and BHA in a dose dependent manner. From the graph, at a 5 μM concentration, compounds (a) and (d) inhibit 86.20% and 93.12% of the activity, respectively, whereas compounds (b), (c), (e) and (f) showed no significant effect on ALP activity. The presence of the N–H group, which can donate hydrogen atoms, in compounds (a) and (d) may contribute to the lipid peroxidation activity. The presence of the methoxy group at the 10th position of the 7 member ring addition to the free N–H group in compound (d) may be responsible for better activity than compound (a). The presence of the methoxy group in the seven member ring may enhance the stability of the nitrogen centred radical due to the electron conjugation effect. The absence of N–

H group in the other compounds (b), (c), (e) and (f) may hinder their lipid peroxidation ability and shows negligible activity in the liposome model.
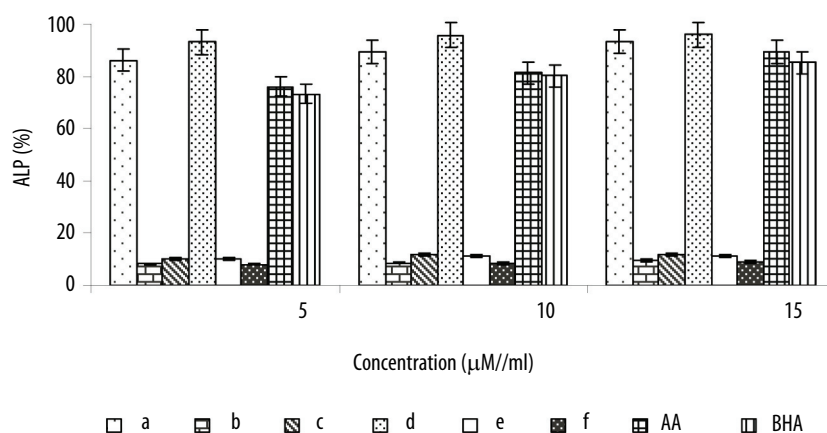

Figure 2: Inhibition of lipid peroxidation (%) in the liposome model system by 5H-dibenz[b,f]azepine and its five analogues at different concentrations (5, 10 and 15 μM/ml). Values represent means ± SD (n = 3).

The polyunsaturated fatty acids (PUFA) of human LDL were oxidised, and the MDA formed was estimated using the TBA method. The antioxidant activity of compounds against human LDL oxidation at different concentrations is shown in the Figure 3. Compounds (a) and (d) showed 85.44% and 92.13% protection at 5 μM level, 92.94% and 94.76% protection at 10 μM level, and 94.69% and 96.39% protection at 15 μM respectively, 6 hr after the induction of oxidation. The results indicate a dose dependent effect of the compound against LDL oxidation. Compounds (b), (c), (e) and (f) showed less activity on human LDL oxidation, whereas compounds (a) and (d) contain free amino groups (N–H bond) that can quench the radical and may inhibit the LDL oxidation.[11,25] Introducing the electron donating group OCH_3 on the seven member ring of compound (a) leads to a considerable increase in the antioxidant activity of compound (d). In the case of compounds (b), (c), (e) and (f), the absence of free N–H and –OCH_3 groups may be responsible for the lower antioxidant capacity on human LDL oxidation. Hence, in this assay, compounds (a) and (d) increase and stabilise the antioxidant activity compared to the other compounds at different time intervals. The percentage inhibition of LDL oxidation for the standards like BHA and AA was also determined and compared with those of the synthesised compounds (Fig. 3). The antioxidant activity of BHA and AA was still lower than that of the compounds (a) and (d). In general, the antioxidant activity on human LDL oxidation observed in the present study was in the following order: (d) > (a) > AA > BHA > (b) > (c) > (f) > (e). These results predict that the
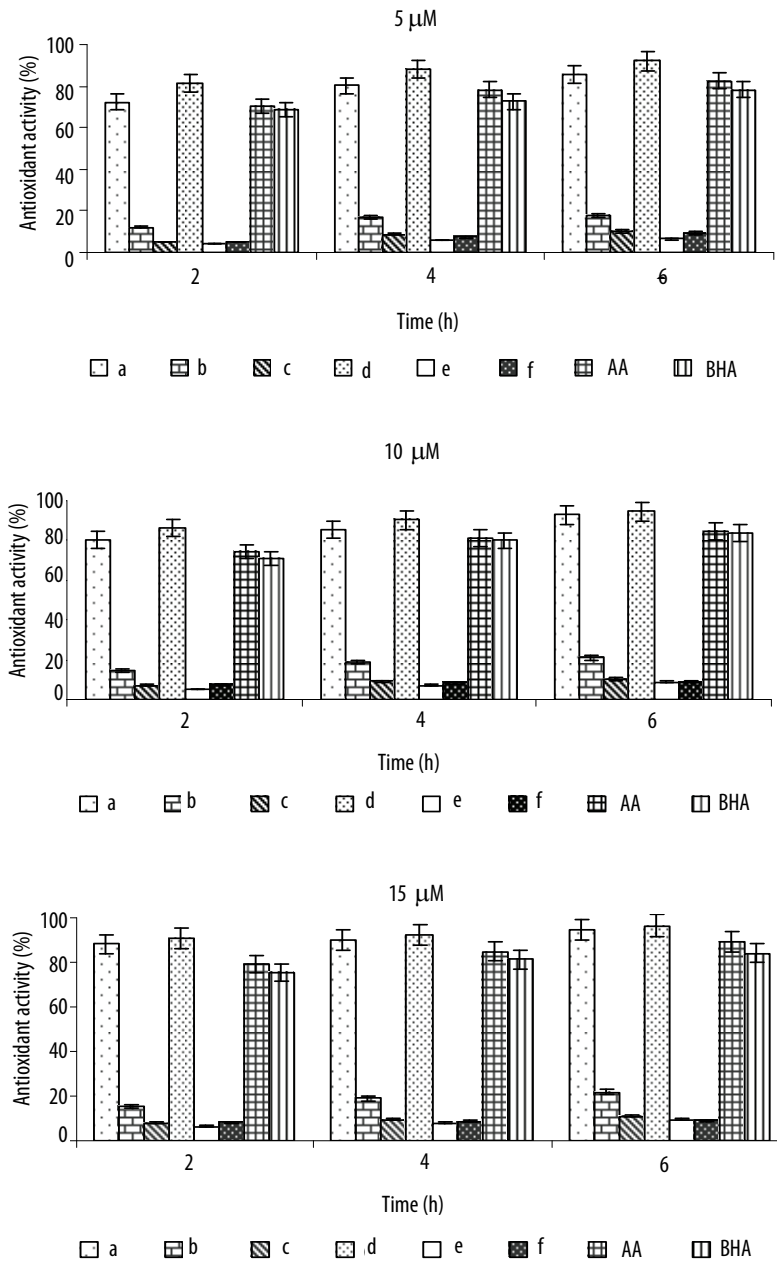
Figure 3: Antioxidant activity (%) of 5H-dibenz[b,f]azepine and its five analogues on human LDL oxidation at different concentrations (5, 10 and 15 μM/ml of LDL). Values represent means ± SD (n = 3).

antioxidant activity of 5H-dibenz[b,f]azepine and its derivatives on human LDL oxidation and lipid peroxidation activity in the liposome system could be related to their direct radical scavenging properties.

4.   CONCLUSION

In the present study, we have successfully synthesised 5H-dibenz[b,f]azepine and its analogues with slight changes in the reagents and conditions with good yield, and the evaluation of in vitro antioxidant activity for the synthesised compounds was also performed. From the results, we can substantiate that the most active compounds like 5H-dibenz[b,f]azepine (a), and its derivative 10-methoxy-5H-dibenz[b,f]azepine (d), have promising antioxidant activity against lipid peroxidation through scavenging free radicals both in the liposome model system and human LDL oxidation assays. Their activity was comparatively better than the standards like BHA and AA. It is conceivable from the studies that the tricyclic amines, i.e., 5H-dibenz[b,f]azepine and some of its analogues, are effective in their antioxidant activity properties. This study provides the theoretical information for medicinal development and supplies some in vitro methods for quickly optimising drugs; our study indicates that 5H-dibenz[b,f]azepine and some of its derivatives can be used as a source of synthetic antioxidants, which could contribute to health benefits. Studies on the antioxidant activities of newly synthesised compounds bearing different functional groups are in progress.

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