Effective attenuation of atrazine-induced histopathological changes in testicular tissue by antioxidant N-phenyl-4-aryl-polyhydroquinolines

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Abstract

Some of the environmental toxicants acting as endocrine disruptors have been associated with health hazards in human and wildlife by modulating hormonal actions. Atrazine, a strong endocrine disruptor, induces detrimental effects on gonads in male and female, and causes impairment of fertility and developmental problems as well as sex alterations. Atrazine decreases the activities of antioxidant enzymes and thus responsible for oxidative stress. Natural antioxidants have shown ability to reduce/slow down the apoptotic effect of atrazine on testicular tissue. In the present study, some N-phenyl-4-aryl-polyhydroquinolines bearing phenolic or/and alkoxy group(s) (6a–6g) were synthesized and evaluated for antioxidant activity in four different assays. Three best compounds (6e–6g) were studied for their ameliorative effect on testicular tissue supplemented with atrazine in vitro.

Keywords

Antioxidant activity, atrazine, endocrine disruptors, N-phenyl-4-aryl-polyhydroquinolines, ROS, testicular tissue

Introduction

There is a growing concern among the scientific community, policy makers as well as general public about the adverse impact on health, in general, and reproductive potential, in particular, of a wide range of chemicals released in the environment as herbicides, fungicides, insecticides, etc.¹² Some of these environmental toxicants strongly act as endocrine disruptors with the potential to alter hormonal action within the body. To cite a few, insecticides (dichlorodiphenyltrichloroethane) DDT and endosulfan, herbicide atrazine, fungicide vinclozolin, various polychlorinated biphenyls used as dielectric and coolant fluids (in transformers, capacitors, electric motors), bisphenol A used in plastics and epoxy resins, polybrominated diphenylethers used as flame retardant, a variety of phthalates as a plasticizers, etc. are some of the commonly accessible endocrine disruptors. Most of these toxicants have been banned in developed countries for agricultural and household purposes due to continuous revelation of their side effects. Unfortunately, they are still being used in developing countries because of their low cost, easy availability as well as the absence of safer and cheaper alternatives. These chemicals are entering into animal and human body through food and drinking water, and their chronic exposure is associated with serious detrimental effects on the body system such as increased incidence of various cancer types, deformations of various body parts besides fertility problems, sexual developmental problems such as feminizing of males or masculine effects on females³–⁶. Low concentration of ROS (reactive oxygen species) generated by sperm is believed to mediate the processes of capacitation, hyperactivation and acrosome reaction which are crucial for effective fertilization of an ovum by a sperm⁷. It has been found that endocrine disrupting chemicals disturb the prooxidant/antioxidant system of the cells leading to the generation of free radicals and thus reactive oxygen species. Mammalian spermatozoa, being rich in membrane bound polysaturated fatty acids (PUFA), are more susceptible to ROS attack making them more vulnerable targets of ROS. Mathur and coworkers have noticed that methoxychlor⁸,⁹ and lindane¹⁰ force oxidative stress on testes including spermatozoa of mammals, by affecting the normal activities of oxidative enzymes. Atrazine, which is popularly known as “the 21st century DDT,” has shown the capability of turning male frogs to females at concentrations as low as 2.5 parts per billion¹¹,¹². Atrazine is believed to decrease the activities of antioxidant enzymes superoxide dismutase, catalase (CAT), glutathione peroxidase (GSH-Px), glutathione-s-transferase, etc. Continuous atrazine exposure is also known to cause cancer in mammals including humans¹³, and developmental problems in fishes and amphibians¹⁴. Detrimental effects of atrazine on gonads (testes and ovaries) have been studied in fishes¹⁵, amphibians¹⁶ and laboratory mammals¹⁷. While it had been widely acknowledged that exposure to atrazine leads to endocrine disruption, it remained a matter of unsettled debate for long time. Studies reported that atrazine disrupts the hypothalamic control of pituitary–ovarian function as well as of testes, demonstrating the direct role of central nervous system (CNS)¹⁸,¹⁹ while others believed that there are certain enzymes which are responsible for conversion of an endocrine product into an active hormonal form in the gonads which, when transferred to hypothalamus/pituitary
gland, further controls endocrine system. Unfortunately, these enzymes are inhibited by high doses of atrazine in the gonads\(^1\). Now it is believed that there is involvement of hypothalamo–pituitary–gonadal axis for controlling the whole mechanism and they are interconnected directly and/or indirectly and influence each other\(^2\). However, it is certain that high doses of atrazine slow down the maturation of gonadotrophic system of male as well as female offspring and counteract the gonads’ endocrinial functioning in adults besides causing oxidative stress.

Antioxidants are believed to counteract the harmful effects of ROS and regulate the physiological defense systems and therefore could be useful for prevention or treatment of oxidative-stress related diseases\(^2\). Phenolic derivatives such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), butylated hydroxyquinone (TBHQ), rosemary diphenol, vanillin, syringaldehyde, flavanoids, tannins, phenolic acids, etc. are ubiquitous antioxidants possessing good antioxidant activity. Besides antioxidant potential, alkyl esters of gallic acid are also found to be potent antitumour agents acting by the induction of apoptosis\(^2\). Lipophilic antioxidants based on galloyl–cinnamic hybrids have shown synergistic effect, acting as chain breaking antioxidants in biomembranes and other type of lipid systems\(^2\). Vitamin therapy is also playing nowadays a decisive role in attenuating the oxidative stress caused by various endocrine disruptors e.g. vitamin E to ameliorate atrazine-induced oxidative stress in liver\(^1\) and testicular tissue\(^2\). Quercetin, a natural flavanoid found to exhibit significant protection of sertoli germ cells against atrazine-induced oxidative damage\(^2\). The very fact that the treatment of oxidative stress produced by atrazine or other endocrine disruptors on various body parts especially gonads is mainly limited to natural products prompted us to design some new molecules in search of an alternative. Quinoline\(^1\) skeleton bearing secondary amine group has been tested in recent past specifically for antioxidant activity and has shown promising results\(^2\) e.g. 1,2-dihydro-2,2,4-trimethylquinoline is used in plastic industry and ethoxiquine in fish meal, animal feeds, spices, etc. Polyhydroquinolines bearing phenolic group alone or in combination with alkoxy groups could prove to be a useful scaffold, led us to synthesize some novel N-phenyl-4-aryl-polyhydroquinolines for testing their antioxidant activity. Compounds showing better antioxidant potential were further evaluated for their ameliorative effect on atrazine-induced oxidative stress in testicular tissue of goat (Capra hircus). To the best of our knowledge, this is in itself the first study of laboratory synthesized molecules towards attenuating the oxidative stress produced by atrazine on testicular tissue, particularly on a domestic animal.

**Materials and methods**

All reactions were carried out under atmospheric pressure. Melting points were determined in open capillaries in an electrical melting point apparatus and were uncorrected. IR spectra were recorded on ABB MB 3000 DTGS IR instrument using the KBr pellet technique. The \(^1\)H NMR and \(^13\)C NMR spectra were recorded on Bruker NMR spectrometer at 300 and 75.5 MHz, respectively. The \(\delta\) values are given in ppm relative to tetramethylsilane (TMS) as internal standard (for \(^1\)H and \(^13\)C NMR). Mass spectra were recorded on JEOL-AccuTOF JMS-T100LC mass spectrometer having a DART (Direct Analysis in Real Time) source in ES\(^+\) mode. The purity of the compounds was checked by \(^1\)H NMR and thin layer chromatography (TLC) on silica gel plates using a mixture of chloroform and methanol as eluent. Iodine or UV lamp was used as a visualization agent. All solvents were dried and/or purified according to the standard procedures prior to use. All reagents employed in the present work were purchased from commercial suppliers and used without further purification. Abbreviations ‘s’ for singlet, ‘d’ for doublet, ‘m’ for multiplet, ‘ex’ for exchangeable proton (detected by disappearance of signal upon D\(_2\)O addition) are used for NMR assignments and ‘s’ for strong, ‘m’ for medium for IR assignments. ‘d’ stands for decomposition in melting point data.

**Synthesis**

**General procedure for synthesis of 3-phenylamino-5,5-dimethylcyclohex-2-ene\(^1\)**

An ethanolic solution (50 mL) of dimedone (I, 42.8 mmol) and aniline (2, 42.8 mmol) was refluxed for 6–7 h. On reaction completion, it was reduced to half of its volume and cooled to room temperature. Yellow crystalline solid separated out which was filtered, washed with cold ethanol (5 mL) and crystallized from ethanol to afford 3-phenylamino-5,5-dimethylcyclohex-2-ene (3). Yield: 80%; m.p. 180–182 °C; Lit.\(^2\) m.p. 184–185 °C. IR (KBr) cm\(^{-1}\): 3232 (m, N–H stretch), 1595 (m, C–H bend); 1H NMR (300 MHz, DMSO-\(d_6\)): \(\delta\) 8.79 (s, ex, 1H, NH), 7.34–7.39 (m, 2H, Ar), 7.10–7.19 (m, 3H, Ar), 5.31 (s, 1H, =CH), 2.38 (s, 2H, CH\(_2\)), 2.05 (s, 2H, CH\(_2\)), 1.02 (s, 6H, 2 \(\times\) CH\(_3\)).

**Representative protocol for synthesis of N-phenyl-4-aryl-polyhydroquinolines (6a–6g)**

An ethanolic solution (25 mL) of 3-phenylamino-5,5-dimethylcyclohex-2-ene (3, 2.79 mmol), appropriate arylaldehyde (4, 2.79 mmol) and malononitrile (5, 3.35 mmol) containing 4–5 drops of triethylamine was refluxed for 10 h. Solution slowly turned reddish-orange which indicated reaction progression. On reaction completion, solution was reduced to half and cooled to room temperature. Solid started separating out or otherwise added small amount (5–10 mL) of ethyl acetate to the concentrated reaction mixture for solid separation which was filtered, washed with ethyl acetate (10 mL) followed by water (30 mL), dried and crystallized from EtOH:EtOAc (1:1) to afford N-phenyl-4-aryl-polyhydroquinolines (6) as yellow- or orange-coloured solid in moderate yield.

2-Amino-4-(2-hydroxyphenyl)-7,7-dimethyl-5-oxo-1-phenyl-1,4,5,6,7,8-hexahydro-3-quinolinecarbonitrile 6a

Yield: 85%; m.p. 286–288 °C (d); IR (KBr) cm\(^{-1}\): 3441 (m, O–H stretch), 3348 (m, N–H stretch), 1595 (m, N–H bend); 1H NMR (300 MHz, DMSO-\(d_6\)): \(\delta\) 9.39 (s, ex, 1H, NH), 7.34–7.39 (m, 2H, Ar), 7.10–7.19 (m, 3H, Ar), 6.99 (t, 1H, J = 7.2 Hz, Ar), 6.76 (d, 2H, J = 7.8 Hz, Ar), 5.09 (s, ex, 2H, NH\(_2\)), 4.71 (s, 1H, CH\(_2\)), 2.16–2.23 (m, 2H, CH\(_2\)), 1.94–2.00 (m, 1H, CH\(_2\)), 1.66–1.71 (m, 1H, CH\(_2\)), 1.08 (s, 3H, CH\(_3\)), 1.02 (s, 6H, 2 \(\times\) CH\(_3\)).
2-Amino-4-(3-hydroxyphenyl)-7,7-dimethyl-5-oxo-1-phenyl-1,4,5,6,7,8-hexahydro-3-quinolinecarbonitrile 6b

Yield: 75%; m.p. 254–256°C; IR (KBr) cm⁻¹: 3464 (m, O–H stretch), 3235 (m, N–H stretch), 2176 (s, C≡N stretch), 1651 (s, C=O stretch), 1597 (s, C=C stretch), 1489 (m, N–H bend); ¹H NMR (300 MHz, DMSO-d₆): δ 9.32 (s, ex, 1H, OH), 7.58–7.62 (m, 3H, Ar), 7.38 (d, 2H, J = 6.6 Hz, Ar), 7.10 (t, 1H, J = 8.1 Hz, Ar), 6.70 (d, 2H, J = 8.4 Hz, Ar), 6.57 (d, 2H, J = 7.2 Hz, Ar), 5.29 (s, ex, 2H, NH₂), 4.36 (s, 1H, CH), 1.98–2.03 (m, 1H, CH₂), 1.66–1.71 (m, 1H, CH₂), 0.83 (s, 3H, CH₃), 0.75 (s, 3H, CH₃); ¹³C NMR (75.5 MHz, DMSO-d₆): δ 195.4 (CO), 151.5, 150.2, 147.8, 145.5, 138.1, 136.8, 130.6, 130.3, 122.0, 119.3, 115.8, 112.6, 61.9, 49.8, 41.4, 35.9, 32.2, 29.6, 26.5; DART-MS: m/z 430.26 (M+H)⁺, C₂₆H₂₇N₃O₄H⁺ calcd. 430.20.

2-Amino-4-(4-hydroxy-3-methoxyphenyl)-7,7-dimethyl-5-oxo-1-phenyl-1,4,5,6,7,8-hexahydro-3-quinolinecarbonitrile 6e

Yield: 85%; m.p. 268–270°C; IR (KBr) cm⁻¹: 3444 (m, O–H stretch), 3235 & 3294 (m, N–H stretch), 1612 (s, C≡N stretch), 1489 (s, N–H bend); ¹H NMR (300 MHz, DMSO-d₆): δ 8.19 (s, ex, 1H, OH), 7.57–7.60 (m, 3H, Ar), 7.32 (d, 2H, J = 6.3 Hz, Ar), 6.47 (s, 2H, Ar), 5.29 (s, ex, 2H, NH₂), 4.39 (s, 1H, CH), 3.74 (s, 6H, 2×OCH₃), 2.19–2.27 (m, 2H, CH₂), 2.00–2.06 (m, 1H, CH₂), 1.66–1.72 (m, 1H, CH₂), 0.89 (s, 3H, CH₃), 0.76 (s, 3H, CH₃); ¹³C NMR (75.5 MHz, DMSO-d₆): δ 195.4 (CO), 151.5, 150.4, 148.3, 137.1, 136.8, 134.4, 130.7, 130.2, 122.0, 112.4, 112.6, 56.5, 56.3, 49.8, 41.5, 36.3, 32.2, 29.7, 26.4, 19.0; DART-MS: m/z 446.26 (M+H)⁺, C₂₆H₂₇N₃O₄H⁺ calcd. 446.20.

In vitro antioxidant assays

DPPH radical scavenging activity

Scavenging activity of compounds against DPPH radical was assessed according to the method of Blois with some modifications. Briefly, 2 mL of each compound in EtOAc (1 mM) was mixed with 2 mL of DPPH methanol solution (0.1 mM). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. Ascorbic acid (1 mM) and BHT (1 mM) were used as references. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ DPPH radical scavenging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where Absₜₚₜ is the absorbance of DPPH radical in EtOAc (ethyl acetate) and Absₜₚ is the absorbance of DPPH radical solution mixed with sample. All determinations were performed in triplicate (n = 3).

ABTS⁺ radical scavenging activity

For ABTS⁺ assay, the procedure followed the method of Re et al. with some modifications. The stock solutions included 7 mM ABTS⁺ solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The reaction was then diluted by mixing 2 mL of ABTS⁺ solution with 30 mL methanol to obtain an absorbance of 0.706±0.01 units at 734 nm using a spectrophotometer. A fresh ABTS⁺ solution was prepared for each assay. 0.1 mL of compound in EtOAc (1 mM) was allowed to react with 2 mL of the ABTS⁺ solution, and the absorbance was taken at 734 nm after 2 min. The ABTS⁺ scavenging capacity of the compound was compared with that of BHT and ascorbic acid and percentage inhibition calculated as:

$$\% \text{ ABTS⁺ radical scavenging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$
where \( \text{Abs}_{\text{control}} \) is the absorbance of ABTS\(^+\) radical in methanol and \( \text{Abs}_{\text{sample}} \) is the absorbance of an ABTS\(^+\) radical solution mixed with sample. All determinations were performed in triplicate \((n = 3)\).

**Ferric reducing/antioxidant power (FRAP)**

The ferric reducing ability of plasma (FRAP) assay was done according to the method described by Benzie and Strain\(^{38} \) with some modifications. The stock solutions included 300 mM acetate buffer \((3.1 \text{~g} \text{ C}_2\text{H}_3\text{NaO}_2 \times 3\text{H}_2\text{O} \text{ and 16 mL C}_3\text{H}_6\text{O}_2)\), pH 3.6, 10 mM TPTZ solution in 40 mM HCl and 20 mM FeCl\(_3\) \( \times 6\text{H}_2\text{O} \) solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL FeCl\(_3\) \( \times 6\text{H}_2\text{O} \) solution and then warmed at 37 \( ^\circ \text{C} \) before using. The working solution was added to the samples in triplicate \((n = 3)\).

**Antioxidant activity in linoleic acid system by the ferric thiocyanate (FTC) method**

The antioxidant activity of compounds against lipid peroxidation was measured through ammonium thiocyanate assay, as described by Mistuda et al.\(^{39} \) with some modifications. The reaction mixture, containing 0.2 mL of 1 mM compound in EtOAc, 0.2 mL of linoleic acid emulsion \((25 \text{mg/mL in 99\% ethanol})\) and 0.4 mL of 50 mM phosphate buffer \((\text{pH} 7.4)\), was incubated in the dark at 40 \( ^\circ \text{C} \). A 0.1 mL aliquot of the reaction solution was then added to 3 mL of 70\% \((v/v)\) ethanol and 0.05 mL of 30\% \((w/v)\) ammonium thiocyanate. Precisely 3 min after the addition of 0.05 mL of 20 mM ferrous chloride in 3.5\% \((v/v)\) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution \( \text{(without compound)} \) reached maximum value. BHT and ascorbic acid were used as references. All determinations were performed in triplicate \((n = 3)\).

**Cytotoxic assays**

**Collection of materials.** Goat \((\text{Capra hircus})\) testes were procured from the slaughter houses around Kurukshetra \((26^\circ6^\prime\text{N}, 76^\circ5^\prime\text{E})\), India, and brought to laboratory in normal saline at 4 \( ^\circ \text{C} \). Then the testes were decapsulated, cut into smaller pieces \((1 \text{ mm}^3)\) and further processed for testicular tissue culture\(^{40} \).

**Reagents/Chemicals.** Atrazine, DMSO, phosphate buffered saline \((\text{PBS})\), DMEM and antibiotics \((\text{penicillin, streptomycin})\) were used.

**Testicular tissue culture in vitro.** The testicular tissues \((1 \text{ mm}^3)\) were washed three times with PBS and then cultured in DMEM medium supplemented with antibiotics \((200\text{-unit having concentration of penicillin 100IU/mL and streptomycin 100IU/mL})\) in CO\(_2 \) incubator \((5\% \text{ CO}_2, 95\% \text{ humidity, 38} \ ^\circ \text{C})\). The testicular tissues were incubated with 10 \( \mu \text{m}\) of each tested compound in DMSO along with 100 nmol/mL concentration of atrazine in DMSO. Harvesting was done after 6 h of culture duration. Then the harvested testicular tissue cultures were washed with PBS and fixed in alcoholic Bouin’s fixative for 24 h. Following dehydration in series of ethanol grades, the testicular tissues were cleared in xylene and embedded in paraffin wax. The paraffin blocks were prepared, and sections were cut at 5 \( \mu \text{m}\) thickness. These sections were then stained with haematoxylin and eosin, and were observed under Olympus microscope \((\text{Japan})\).

**Results and discussion**

**Chemistry**

The synthesis of \(N\)-phenyl-4-aryl-polyhydroquinolines \(6\) was achieved in two steps starting from aniline \((2)\) involving one pot multi-component reaction strategy according to Scheme 1. 3-Phenylamino-5,5-dimethylcyclohex-2-enone \((3)\) was obtained by condensation of dimedone \((1)\) with aniline \((2)\) in reflowing ethanol. Treating 3 with phenolic aldehydes \((4)\) and malononitrile \((5)\) in a molar ratio of \((1:1:2)\) in reflowing ethanol for 10 h containing catalytic amount of triethylamine yielded the corresponding \(N\)-phenyl-4-aryl-polyhydroquinolines \(6\) as target compounds in moderate yield. 3-Phenylamino-5,5-dimethylcyclohex-2-enone \(3\) was characterized by comparing its spectral data as well as melting point\(^{34} \). The structure of \(6a-6g\) was assigned on the basis of its FT-IR, \(^1\text{H}\text{ NMR}\) and \(^1\text{C}\text{ NMR}\) spectral data and was confirmed on the basis of mass spectra \((\text{DART-MS})\). A characteristic absorption band in the region 2176–2206 cm\(^{-1}\) was assigned to \(\text{C}\equiv\text{N}\) stretching in IR spectra of all the compounds besides displaying other important absorption bands at 3441–3472 cm\(^{-1}\) corresponding to \(\text{O–H}\) stretching, 3325–3348 cm\(^{-1}\) due to \(\text{N–H}\) stretching and 1636–1651 cm\(^{-1}\) due to \(\text{C=O}\) stretching. Phenolic OH was further confirmed by the presence of an exchangeable singlet in the region \(\delta 8.19–9.75\) in \(^1\text{H}\text{ NMR}\) spectra. NH\(_2\) protons and methine \((\text{CH})\) proton of the dihydropyridine skeleton were assigned in the region \(\delta 5.09–5.33\) and \(\delta 4.36–4.89\), respectively, whereas two methyl groups were confirmed in the aliphatic region at \(\delta 0.87–0.89\) and \(\delta 0.72–0.83\). Methoxy groups in \(6e-6g\) were clearly assigned in the region \(\delta 3.74–3.75\). A multiplet–triplet pattern due to two and three protons at \(\delta 3.94–4.04\) and \(\delta 1.33\), respectively, present in compound \(6f\) confirmed the presence of –OCH\(_3\)C\(_3\)H\(_2\) group. Other appropriate signals were easily assigned in aromatic and aliphatic regions.

**In vitro antioxidant activity**

It has been reported that sometimes compounds show antioxidant activity \(\text{in vitro}\) in extracellular environment but unfortunately, their efficacy in the intracellular environment is often decreased, probably due to their poor solubility as well as poor permeability across the cell membrane\(^{41,42} \). Therefore, to make an effective antioxidant compound in a biological context, solubility in different media as well as transport across the cell membrane are extremely important besides other governing factors. That is why various methods are employed to check the antioxidant potential of a compound which vary in environment as well as generation of free radicals\(^{31,42} \). In our study, we used three different simple redox-based assays, each having its own uniqueness as well as importance but all involving one redox reaction with the oxidant, for measuring the reducing capacity of the test compounds. Among these assays, the DPPH assay is influenced by the kinetic behaviour of the antioxidant compound, while the FRAP and ABTS\(^{+}\) assays are carried out at acidic and neutral conditions, respectively\(^{43} \). Importance of the DPPH method lies in the fact that this method is independent of compound’s polarity, while the ABTS\(^{+}\) method is applicable for both lipophilic and hydrophilic compounds\(^{42} \). The FRAP assay is inexpensive, reagents are simple to prepare, the results are highly reproducible, and the procedure is straightforward and speedy\(^{38} \). Other assay employed in this study has been selected to evaluate the antioxidant activity of the polyhydroquinolines under
investigation in linoleic acid system by FTC method. The radical scavenging and total antioxidant activity of compounds (1 mM) were compared with those of ascorbic acid and BHT at the same concentration and expressed as % inhibition against DPPH, ABTS and mM TE, respectively (Table 1).

DPPH and ABTS assays are based on the ability of the test compounds to scavenge synthetic-free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. ABTS or DPPH radical scavenging methods are common spectrophotometer procedures for determining the antioxidant capacities of components. In DPPH method, the DPPH radical is used as a substrate to evaluate free radical scavenging activities of compounds 6a–6g. It involves the reaction of specific antioxidant with a stable-free radical DPPH. As a result, there is reduction of DPPH concentration by the antioxidant that leads to a decrease in the optical absorbance of DPPH that is detected by spectrophotometer at 517 nm. All the seven compounds (6a–6g) tested showed antioxidant activity less than ascorbic acid but three compounds 6e–6g showed activity comparable to BHT.

The ABTS activity of all the compounds 6a–6g was found to be higher than BHT (15.3 ± 0.7%), and the four compounds 6a, 6c–6g were found to exhibit excellent activity (>90%) while the other two compounds 6d and 6c showed moderate activity when compared with ascorbic acid (>100%).

In FRAP assay, reduction of ferric tripyridyl triazine (Fe³⁺-TPTZ) complex to ferrous form (which has an intense blue color) at low pH can be monitored by measuring the change in absorbance at 593 nm. The change in absorbance is therefore directly related to the combined or “total” reducing power of the electron donating antioxidants present in the reaction mixture. Only three compounds (6e–6g) could manifest FRAP activity, showing capability to inhibit free radical chain reaction by donating a hydrogen atom. Compound 6g showed much higher FRAP activity than the remaining two compounds and BHT which was comparable to ascorbic acid.

During linoleic acid peroxidation, peroxides are formed and these compounds oxidize Fe²⁺ to Fe³⁺. The Fe³⁺ ion forms a complex with SCN⁻, which has a maximum absorbance at 500 nm. Thus, a high-absorbance value was an indication of high-peroxide formation. This method measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of oxidation. All tested polyhydroquinolines hindered the oxidation of linoleic acid for all 5 days, and the antioxidant activity of compound 6g was comparable to that of BHT. Ascorbic acid lost its peroxide quenching ability very soon (after 2 days) and started demonstrating pro-oxidative effect at this concentration from the 3rd day (Figure 1). Antioxidant activity data can be seen in supplementary information (Table 2).

A common structure–activity relationship (SAR) was clearly noticed amongst tested polyhydroquinolines in the sense that antioxidant potential decreases as the position of phenolic hydroxyl group varies from ortho to para i.e. away from (hydro)quinolinic system. It means that the synthesized system could show better antioxidant potential when phenolic OH is present at ortho-position. Addition of (5)-Cl group in 6a, i.e. compound 6d, showed decrease in antioxidant activity. Combination of (para) phenolic hydroxyl group with alkoxy group was also studied and was found that (4-)phenolic hydroxyl flanked by two (3- & 5-) methoxy groups, i.e. 6g, showed...
enhanced antioxidant activity as compared to phenolic group associated with single methoxy group as in 6e. Changing the methoxy group (6e) to ethoxy (6f) showed a slight decrease in antioxidant potential.

In vitro ameliorative study on atrazine-induced oxidative stress on testicular tissue

In the present investigation, three compounds 6e–6g showing promising antioxidant activity in all the four employed methods were further evaluated for their ability to ameliorate the histopathological changes induced by atrazine in testicular tissue of goat in vitro. The concentration of synthesized compounds and atrazine was kept at 10 μmol/mL and 100 nmol/mL (DMSO), respectively, in the media for carrying out the experiments. Testicular tissue sections (5 μm) stained with haematoxylin and eosin revealed normal histoarchitecture of the seminiferous tubules in control group under microscope (×1000) (Figure 1). Seminiferous tubules were packed with loose connective tissue having blood vessels. Normal Leydig cells were present outside the seminiferous tubules. Sertoli cells and all types of the germ cells were present in the seminiferous tubules well arranged in the specific manner. Spermatagonia were present at the basal portion and next to these, spermatoocytes occupied the middle portion. Spermatids formed the next layer and then mature elongated spermatозoa were present toward the lumen of the seminiferous epithelium. Sertoli cells were having broad basal portion with round or oval nuclei resting on the basal lamina and its cytoplasmic part with irregular extensions scattered in between the germ cells up to the lumen of the seminiferous tubules (Figure 2a).

In the experimental group, treated with atrazine having concentration 100 nmol/mL in the culture medium, histomorphological alterations were revealed in testicular tissues after 6 h of atrazine exposure. Many atretic changes were noticed in different types of germ cells such as hyalinization, margination of chromatin, pinching off of the nuclear material, pynotic nuclei, crescent shaped nuclei, chromolysis, vacuolization, fragmentation of nuclei, decondensation of nuclear materials, appearance of meganucleated cells, disruption in the normal arrangement of cells, etc. (Figure 2b). Degenerating Leydig cells were also observed in the testicular sections. Basal lamina was also detached from the underlying cells in few portions of seminiferous tubules (Figure 2b). Supplementation of compounds 6e–6g (10 μmol/mL) separately in next three experiments along with the exposure of atrazine promisingly showed antioxidant-type attenuation/prevention of degenerating changes induced by atrazine in testicular tissue. Reduction/delaying in atretic changes were clearly observed in each case such as decline in chromolysis, condensation, fragmentation and vacuolization (Figure 2c–e).

The results of the present investigation clearly demonstrated that atrazine-induced atretic changes like vacuolization, chromolysis, pynosis, fragmentation, hyalinization, condensation were dramatically reduced when the atrazine-cultured testicular tissue was simultaneously supplemented with compounds 6e–6g separately, thereby indicating the protecting effect of these compounds against atrazine-induced cytotoxicity. Although all the three compounds (6e–6g) showed ameliorative effect, protecting ability (delaying in degenerative changes) of 6e was found to be much higher followed by 6g and then 6f.

Conclusions

The objective of the present work was to investigate some new organic molecules which could have potential ability to act like natural antioxidants (Vitamin C & E) and attenuate the apoptotic effect of atrazine on testicular tissue in vitro. We could find N-phenyl-4-aryl-polyhydroquinolines as a potential scaffold which could be synthesized by following one pot multi-component reaction strategy and might possess antioxidant activity comparable to natural antioxidants. Seven N-phenyl-4-aryl-polyhydroquinolines (6a–6g) were synthesized by treating 3-phenylamino-5,5-dimethylcyclohex-2-enone with various phenolic aldehydes and malononitrile in refluxing ethanol containing catalytic amount of triethylamine. All seven target compounds were tested in vitro for antioxidant activity by three different assays (DPPH, ABTS+ and FRAP) and by ferric thiocyanate.
method. Three compounds (6e–6g) exhibited better antioxidant activity in all the assays which were further tested for their ameliorative effect on degenerative changes in testicular tissue induced by atrazine in vitro and showed promising results. Data suggested that introduction of alkoxy group significantly enhanced the antioxidant activity of polyhydroquinolines and thus ameliorative effect on atrazine-induced histopathological changes in testicular tissue.

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**Declaration of interest**

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