BIOACTIVITIES OF HOLMIUM(III) AND GADOLINIUM(III) COMPLEXES OF THYMOQUINONE

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ABSTRACT. Chemotherapeutic agents which are the main stay in cancer treatment are toxic with numerous contrary side effects. A number of chemical, physical, and computational techniques were applied to synthesize and elucidate the structural and functional characterization of the new designed bioligands and their metal complexes. Besides, several biological techniques for developing therapeutics and diagnostics agents of these new designed materials were used. The trivalent holmium(III) and gadolinium(III) metal complexes of thymoquinone (TQ) were synthesized. Toxicities and other bioactivites were undertaken with existing drug combinations and more effective tumor models will be established. The molecular structures of TQ-metal complexes were elucidated based on particular spectral approaches. The NF-kB (nuclear factor kappa-light-chain enhancer of activated B Cells) luciferase, elastase release, superoxide anion ($O_2^{\bullet-}$) generation, and DPPH (1,1-diphenyl-2-picryl hydrazyl) free-radical scavenging activities of TQ and its synthesized complexes were elucidated and discussed. The core research is to use coordination and organometallic chemistry to design new bioligands and binary, ternary, mixed ligand, multi metal multi ligand complexes pursing a bio target continuously with structure-activity relationships (SARS).

**KEY WORDS**: Thymoquinone, Holmium, Gadolinium, Bioactivities

INTRODUCTION

At the present time, there is a tendency in searching for anticancer substances in the natural sources. These natural sources are usually supposed to be fewer toxic effects and produce negligible side effects [1, 2]. Medications from ordinary sources have been used conventionally

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for thousands of ages in manyplaces of the world [3]. Experts have embattled numerous traditional or common drugs in similar of contemporary medicine to recognize and extract vigorous ingredients for the medication development [4]. Thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone, Scheme 1), a monoterpene molecule is a most prominent constituent phytochemical compound found in black cumin (*Nigella sativa*) with a long history of medicinal use [5]. Clinical studies with thymoquinone (TQ, Scheme 1) in humans had showed its general safety even up to a dose of 10 mg/kg/day with individual few side possessions [6-10].
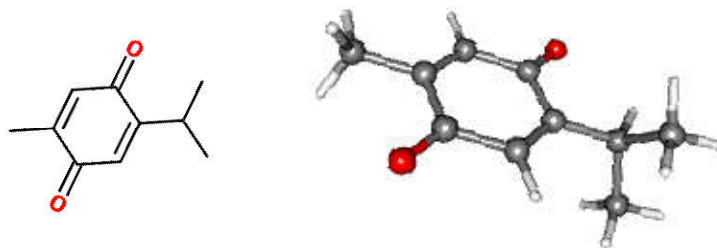
Scheme 1. Molecular structure of TQ and its computer-generated model.

The problem met in clinical trials concerning TQ is its poor bioavailability, leading to little levels in plasma and tissue as shown in most pharmacokinetics research work done in TQ bioligand [11-22]. As well, it's hydrophobic in nature, rapidly eliminated and slowly/poorly absorbed. However, exploring other mechanism to improve the bioavailability of TQ especially chemical modelling may prove as a novel method [11-22]. Furthermore, great lipophilicity of TQ makes poor formulation features. Numerous investigational studies have been done to avoid the pharmacokinetic difficulties of thymoquinone [23-25], and its contrary effect. An extremely promising and advanced approach to deal with the bioavailability matter and to realize even extra various potential health benefits is the usage of metal ion-TQ complex, forming a binary complex formation. Both holmium and gadolinium metals play no known biological role in biological processes, however, their salts and chelates were found to be able to stimulate metabolism in humans. Holmium and gadolinium forms trivalent ions occurring in water-soluble salts with fluorescent properties and toxic to mammals [26, 27]. Though, homium(III) and gadolinium(III) chelates are far less toxic because they carry metal ions over the kidneys and out of the body before the free ion can be released into the body tissues [26, 27]. Moreover, because of their paramagnetic properties, solutions of chelated organic holmium and gadolinium complexes were used as intravenously directed gadolinium/holmium-based magnetic resonance imaging contrast agents in medical magnetic resonance imaging [26, 27].

The role of metal-ligand complex equilibria and the formation of bio-ligand complex is of therapeutic research interest [28-30]. To our knowledge, no work had been reported in using such mechanism in overcoming TQ bioavailability problem. TQ had shown to be safe in human beings. However, the usage of TQ in humans is inadequate owing to its chemical properties and poor membrane diffusion capability. The present study will have aimed at unfolding the possible use of the formed metal bio-ligand as a novel alternative in overcoming problems encountered with TQ in biological system and its therapeutic application. Recently, The stability constants of holmium(III) and gadolinium(III) metal ions-thymoquinone ligand complexation were considered wherein, the protonation constant of thymoquinone and the complex stability constants of each binary metal complex between both this metal ions and thymoquinone ligand (TQ) were determined and stated [31]. The hypothetical calculations of the global dissociation and complex stability constants of each metal complex classes in solutions were anticipated as

the free energy change concomitant with the ligand protonation, dissociation, and metal ion-TQ complex process equilibria by means of density function theory (DFT) designs. The ΔG values established that TQ form complex classes bind both metal ions and turns as chelating ligand. Moreover, the formation of diverse thymoquinone-holmium(III) and gadolinium(III) metal complex systems was established to be spontaneous process and exothermic interaction [31]. In this study, a structural characterization of the synthesized thymoquinone-holmium(III) and gadolinium(III) metal complexes was considered. Studying the antioxidant and antitumor relevance of holmium and gadolinium metal ion complexes of thymoquinone using cytotoxic and free radical scavenging assays was achieved.

EXPERIMENTAL

Chemicals and reagents

All the chemical substances, supplies, and organic solvents used during this research work were of analytical chemical grade and were used without additional purifications such as thymoquinone (Sigma-Aldrish, USA). This TQ material was assessed in triplicate by titration by a carbonate-free solution of standard NaOH chemical substance [31]. This test showed that the mass fraction purity of it was (0.99 ± 0.05). Holmium(III) nitrate pentahydrate $(Ho(NO_3)_3 \cdot 5H_2O;$ 99.99% trace metals basis), and gadolinium(III) nitrate hexahydrate $(Gd(NO_3)_3 \cdot 6H_2O;$ 99.999% trace metals basis) were used supplied from Sigma-Aldrich, USA. The organic solvents, methanol, ethanol, ether were of high purity.

Synthesis of HoTQ and GdTQ metal complexes

Holmium(III) and gadolinium(III) TQ complexes were created according to the next common procedure: An methanolic aliquots (20 mL) of each metal compound salts was added slowly in minor quantities to magnetically stirred methanolic aliquots (20 mL) of TQ till the reaction finished and refluxed at temperature of around 185 ^{o}C for 6 h. At that time, dehydration by pass in the subsequent reaction mixture in closet gases room, resulting in the isolation of solid precipitated complex yields. Then, they were filtered off, washed carefully using diethyl ether and ethanol solution mixture numerous times to eliminate any traces of unreacted starting ingredients and lastly dried in a vacuum desiccator over fused $CaCl_2$ (final yield: 35-55%). Digital Elico Conductivity Bridge meter (Model No. CM-180) was used to measure the molar conductance of the TQ bioligand and its holmium and gadolinium metal-TQ complexes in DMSO organic solutions with a concentration of about 1×10^{-3} mol.dm^{-3} at room temperature by means of a dip-type conductivity cell tailored with a platinum electrode. Vibration infrared spectral (IR) studies of all prepared complexes were verified on a Shimadzu FT-IR 8000 spectrophotometer by means of KBr disc medium in the range 400–4000 cm^{-1} and the spectra were collected with a resolution of 2 cm^{-1} with 15 scans [32].

Cellular bioactivities of the synthesized metal complexes

NF-kB luciferase assay using HSC-T6 C cells

HSC-T6 Cells (around 7.5×10^4 cells/well) were sowed in 24-well micro plates with Fetal Bovine Serum (FBS) free medium at 37 °C for one day earlier transfection. Transfection was achieved with transfection reagent FuGENE®6 transfection reagent (Promega, Madison, USA) rendering to manufacturer's conditions. NFκB-Luc reporter construct (1.2 μg/well) (Cayman Chemical, Michigan, USA) was added to the cells along with plasmid cytomegalovirus-β-galactosidase (CMV-β-gal, 0.22 μg/well; Promega, Madison, USA) and transfection reagent (20

µL/24-well plate). CMV-β-gal helped as an internal control to regularize the transfection efficacy. Subsequently, one day incubation, cell cultures were preserved with tested compounds (TQ bioligand, HoTQ, and GdTQ metal complexes) for half hour. Lipopolysaccharides (LPS) was then added to motivate NF-κB activity for 6 h. Cell lysates were harvested with diluted reporter lysis 5X buffer (Promega, Madison, USA). 24-well plates were added reporter lysis buffer (100 µL/well), and each 20 µL cell lysate assorted with 100 µL luciferin in 96-well plates. Luminescence was identified by microplate lumimometer multilabel counter (Titertek-Berthold, Pforzheim, Germany) [33, 34].

Elastase release estimation

Experimens were done using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as elastase substrate. In brief, afterward supplementation with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 µM), neutrophils (6×10^5/mL) were equilibrated at 37 °C for 2 min and incubated with TQ bioligand and its metal complexes for 10 min. The cells were stimulated using formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) (100 nM) and cytochalasin B (CB) (0.5 µg/mL). The variations in absorbance at *ca.* 405 nm (*p*-nitrophenol) were instently measured to detect elastase release. The results are stated as a percentage of the original rate of elastase release in the formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP)/cytochalasin B (CB)-activated, drug free control arrangements. To test whether the TQ bioligand and its metal complexes display an inhibitory ability to elastase activity, a direct elastase activity assay was done in a cell-free system. Neutrophils (6×10^5/mL) were incubated for 20 min with formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) (100 nM) and cytochalasin B (CB) (2.5 µg/mL) at 37 °C. After that, cells were centrifuged at 1,000 g for 7 min at 4 °C to collect the elastase from the supernatant. Then, the supernatant was equilibrated at 37 °C for 5 min and incubated with or without TQ bioligand and its metal complexes for 10 min. Then, the elastase substrate, MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 µM), was added to the reaction blends. Deviations in absorbance at 405 nm were incessantly detected for 10 min to evaluate the elastase activity [35-36].

Measurement of superoxide anion ($O_2 \bullet -$) generation

The evaluation of $O_2^{\bullet-}$ generation was done using the superoxide dismutase (SOD) inhabitable reduction of ferricytochrome C. Briefly, once supplementation with 0.5 mg/mlferricytochrome C and 1 mM Ca^{2+}, neutrophils ($(4 \text{ or } 10) \times 10^5$ µg/mL) were equilibrated at 37 °C for about 2 min and incubated with TQ bioligand and its metal complexes for 10 min. Cells were initiated with formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) (100 nM) for 10 min. for 5 min. When formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) was used as a stimulant, cytochalasin B (CB) (1 µg/mL) was incubated for 5 min. Before activation by the peptide (FMLP/CB), superoxide anion($O_2^{\bullet-}$) generation by isolated neutrophil fractionation was detected after the addition of 160 µM NADPH to 800 µL of relaxation buffer having 4×10^6 cell equivalents of membrane extract, 1.2×10^7 cell equivalents of cytosol, 2 µM GTP-γ-S, 0.5 mg/mL ferricytochrome C, and 100 µM sodium dodecyl sulfate. To make informal the association of NADPH oxidase components, all ingredients (without NADPH) were incubated at 37 °C for 5 min earlier the addition of NADPH. TQ bioligand and its metal complexes were incubated for 2 min before NADPH oxidase assemblage. Variations in absorbance with the reduction of ferricytochrome C at 550 nm were constantly detected. Calculations were done based on alterations in the reactions with and without superoxide dismutase (SOD, 100 U/ mL) divided by the extinction coefficient for the reduction of ferricytochromec ($\varepsilon = 2.11 \text{ mM}^{-1} \text{ mm}^{-1}$) [37].

DPPH radical scavenging activities

Antioxidant influence of the tested compounds were established using the stable radical, 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH). All tested complex species were prepared in a mixture of neutral saline and DMSO. Around 50 µL of each tested compound was retained in 1 cm cuvettes plus 2 mL methanolic mixtures of DPPH were added. At that time absorbance was detected. The reduction in absorbance was detected at 517 nm continually with data acquirement at 2 s intervals till the absorbance eased. The DPPH absorbance radical without the tested samples (control) was stately every 24 hours. Unusual maintenance was taken in consideration to diminish the loss of free radical activity of the DPPH radical standard solution as suggested. All experiments were done in triplicate. The percentage inhibition of the DPPH radical by gallate solution as standard antioxidant material and all tested compounds and their unique TQ bioligands was calculated based on the following equation: Percent inhibition = $([A_{C(0)} - A_{C(t)}] / [A_{C(0)}]) \times 100$, where $A_{C(0)}$ is the absorbance detected of the control at $t = 0$ min and $A_{A(t)}$ is the absorbance detected of the reaction solution mixture at $t = 16$ min [38].

Statistical analysis

The investigational data were statistically evaluated by a parametric two tailed t test, and calculated probability p values less than 0.05 were found to be a statistically significant. Once more than two investigational sets were examined, an ANOVA test by means of SPSS 17.0 statistical package was similarly used to estimate the statistical significance data.

RESULTS AND DISCUSSION

Physical and chemical characterizations of synthesized complexes

TQ bioligand, as well as its Ho(III), and Gd(III) complexes were exposed to elemental analysis. The elemental chemical and physical analytical data of the complexes showed 1:2 molar ratio (Ho/Gd : TQ). The synthesized complexes have melting points above 300 °C. The molar conductivity values for the synthesized complexes $(1.0 \times 10^{-3}$ mol/mL) were found to be between 32 and 51 $\Omega^{-1}cm^2mol^{-1}$ indicating lightly electrolytic nature, and providing the degree of ionization of the complexes due to the existence of nitrate ions inside the coordination sphere which powerfully maintained with the elemental analysis data. The magnetic susceptibilities (μ_{eff}) of the Ho(III) and Gd(III)-TQ ligand complexes at 25 °C were found to be reliable with noticeable low-spin diamagnetism. The revelation of molecular structures of the prepared Ho(III) and Gd(III)-TQ ligand complexes were supported by IR spectroscopic technique. The prepared Ho(III) and Gd(III)-TQ ligand complexes were found to be stable at 25 °C with two diverse colors (Figure 2), wherein they are partially soluble in D_2O, while, they are soluble in DMSO and DMF organic solvents. The IR spectrum (Figure 1) showed a characteristic stretching band of the carbonyl group of a cyclohexadiene is observed at the wavenumber 1650 cm^{-1}. The band presented at 2967 cm^{-1} relates to the C-H stretching of aliphatic functional groups. The fragile wide band observed at a higher wavenumber (about 3040 cm^{-1}) was assigned to the vinylic C-H in the C = C-H groups. The C = C stretching bands (1640-1675 cm^{-1}) in 1,4-cyclohexadiene cannot be definitely recognized because the strong carboxylic stretching bands in thymoquinone is existing in this frequency range. Careful inspection of the IR spectra of free TQ bioligand and their the Ho(III) and Gd(III)-TQ ligand complexes was done in order to simplify the obligation of these bands in the free TQ bioligand and its holmium and gadolinium complexes (Figure 1).
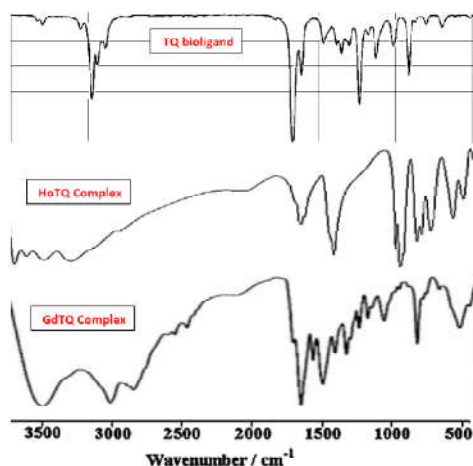
Figure 1. IR spectrum of TQ bioligand and its holmium (HoTQ), and gadolinium (GdTQ).

NF-kB luciferase assay using HSC-T6 cells

The inhibitory effects of the tested TQ and its complexes were evaluated on inflammatory HSC-T6. The most active compound was found to be TQ compound, followed by compounds Ho(TQ), then Gd(TQ) with inhibitory activity (%) equals to 28.4, 14.2, and 13.2, respectively, against HSC-T6 (Table 1). Numerous experimental methods including immunoblot analysis, luciferase reporter gene assay, enzyme assay, over expression of target gene, and immunoprecipitation analysis confirmed that TQ targets interleukin-1 receptor-associated kinase 1 (IRAK1), which is complicated in the activation of together activator protein AP-1 and nuclear factor NF-κB [39]. Furthermore, TQ instantaneously inhibited the induction of both NF-κB- and AP-1-driven luciferase activities [40]. The present results could suggest that several enzymes involved in the induction of the NF-κB and AP-1 pathways are targeted by TQ as well its Ho and Gd metal complexes.

Table 1. Inhibitory ratio of TQ bioligand and its metal complexes on inflammatory HSC-T6cells.

Tested compound	Silymarin (5 μg/mL)	INF-α (5 μg/mL)	TQ (25 μg/mL)	HoTQ (50 μg/mL)	GdTQ (50 μg/mL)
Light intensity	39	109	81	59	43
Inhibitory activity (%)	87	0	28.4	14.2	13.2

Elastase release estimation and detection of superoxide anion (O_2•−) generation

Herein, the inhibitory effects of the TQ bioligand and its metal complexes were assessed on superoxide dismutase (SOD) and elastase release in human neutrophils and gadolinium(III)-TQ complex showed a moderate activity against the inhibition of superoxide anion generation (Table 2) against the inhibition of superoxide anion generation. TQ-holmium(III) complex showed a strong inhibitory effect against elastase release in stimulated human neutrophils. While, TQ and its gadolinium(III) complex showed a moderate inhibitory effect (Table 2) against elastase release in stimulated human neutrophils. Previous *in vivo* studies demonstrated that thymoquinone (TQ), a lipid soluble benzoquinone is a bioflavonoid managing inflammatory-associated diseases in which it reduces articular elastase and myeloperoxidase (MPO) activities, and suppresses the expression of pro-inflammatory cytokines [41].

Table 2. Inhibitory effects of TQ bioligand and its metal complexes on superoxide anion generation and elastase release in FMLP/CB induced human neutrophils.

Tested compounds	Superoxide anion	Elastase release	Superoxide anion	Elastase release
	IC_{50} (µg/mL)		Inhibitory %	
TQ	1.13±0.12	26.67[a]±0.57	15.37[a]±0.74	12.36[b]±0.57
HoTQ	1.64±0.11	1.80±0.13	79.56[c]±2.10	99.55[c]±3.72
GdTQ	16.23[b]±0.34	105.84[c]±3.15	20.09[b]±1.13	6.32±0.78

[a]Percentage of inhibition (Inhibitory %) at 10 mg/mL concentration. Results are presented as mean ± S.E.M. (n = 34). [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ compared with the control value (DMSO).

DPPH free-radical scavenging test

Antioxidant activity is unique of the significant standard tests in pharmaceutical industry to inspect the potency of a tested compound to inhibit the creation of free radical. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a general "steady free-radical" for antioxidant test. The vigorous DPPH radical has a robust absorption band at 517 nm with a violet color in solution. The neutralization of DPPH by each antioxidant tested compound gives band at 517 nm to decline and each solution color develops pale yellow color. From the results obtained for the tested compounds, it was found all TQ complexes showed an antioxidant activity (Figure 2). The cytochrome C system turned out to be inappropriate for the assessment of the antioxidant activity of thymoquinone. Thymoquinone concentration-dependently (250–2000 ppm) stimulated the NADH oxidation of submitochondrial particles in which he thymoquinone-stimulated NADH oxidation was sensitive to inhibitors of the mitochondrial electron transfer [42]. Generally, thymoquinone possesses a moderate antioxidant activity assuming that the mitochondrial respiratory chain is significant for the antioxidant properties of thymoquinone in the cell in contrast to tis Ho and Gd metal complexes.
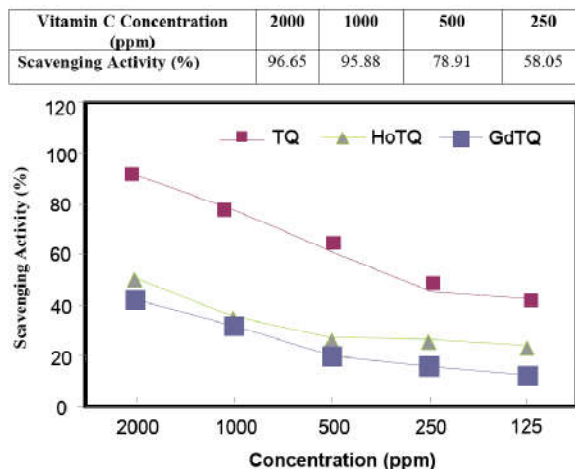
Vitamin C Concentration (ppm)	2000	1000	500	250
Scavenging Activity (%)	96.65	95.88	78.91	58.05

Figure 2. DPPH scavenging activity of TQ bioligand and its metal complexes comparing with vitamin C.

CONCLUSION

The present study aimed at unfolding the possible usage of the formed bio-ligand metal complexes as a novel alternative in overcoming problems encountered with thymoquinone in biological system and its therapeutic application. Thymoquinone had shown to be safe in human beings. However, the use of thymoquinone in humans is limited due to its chemical properties and poor membrane penetration capacity. Thymoquinone is chemically hydrophobic, which causes its poor solubility, and thus bioavailability. Many experimental studies have been conducted to overcome the pharmacokinetic problems of thymoquinone, its adverse effect. A highly promising and innovative approach to deal with the bioavailability issue and to achieve even more diverse potential health benefits is the use of metal thymoquinone complex, forming a complex formation. Yet, discovering other mechanism to increase the bioavailability of thymoquinone particularly chemical modelling may show as a novel method. The role of metal-ligand complex equilibrium and the formation of bio-ligand complex are of therapeutic interest in this study.

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