Guangyu Zhai*, Wei Zhu, Yandan Duan, Wentao Qu and Zitong Yan

Synthesis, characterization and antitumor activity of the germanium-quercetin complex

Abstract: Quercetin-germanium (Ge) (IV) complex was synthesized in the laboratory. The structure and physicochemical properties of the complex were characterized utilizing thermal and spectroscopic (UV-vis, IR and ^{1}H NMR) analysis. The antitumor activity of quercetin-Ge (IV) compound was evaluated by the MTT method. The complex was subjected to biological tests *in vitro* using four tumor cell lines (PC-3, Hela, EC9706 and SPC-A-1). The quercetin-Ge (IV) complex showed significant cytotoxicity against four tumor cell lines.

Keywords: antitumor; flavonoids; quercetin-germanium (Ge) (IV) complexation; spectroscopy.

***Corresponding author: Guangyu Zhai**, College of Nursing, Zhengzhou University, 450052 Zhengzhou, China, e-mail: zhaiguangyu1@sina.com
Wei Zhu: College of Pharmaceutical Sciences, Zhengzhou University, 450001 Zhengzhou, China
Yandan Duan: College of Nursing, Zhengzhou University, 450052 Zhengzhou, China
Wentao Qu: College of Pharmaceutical Sciences, Zhengzhou University, 450001 Zhengzhou, China
Zitong Yan: College of Pharmaceutical Sciences, Zhengzhou University, 450001 Zhengzhou, China

Introduction

Free radicals which can cause lipid peroxidation injury of cell and organelle membranes are reactive molecules due to the presence of one or more unpaired electrons. Reasons for various diseases seem to be at least partly connected to the free radicals in the body (Agnes et al., 2008). Quercetin, referred to as polyphenols, is one of the most common flavonoids present in nature. It has been associated with a variety of pharmacological properties, including scavenger and antioxidant activities, which has been the focus of attention of many researchers (Erlund, 2004). Quercetin is abundant in many plants and in plant-derived foods such as fruits, vegetables, drinks and medicinal herbs (Trevor and Kathryn, 2000). Researchers also suggest that quercetin has a wide range of biological properties such as antiviral, anti-inflammatory, antitumor, antiasthma and antiallergic (Moskaug et al., 2004). However, clinical uses of quercetin are few presumably because of its unfavorable physicochemical as well as pharmacokinetic properties. The biological activity of flavonoids is related to their chemical structures, so its bioavailability and water solubility can be improved by structure modification (Chen et al., 2010).

Hydroxyl and oxo groups present in a quercetin structure have the ability to form complexes with various metal ions (Bravo and Anacona, 2001). A number of researchers focused their attention on the complexes of quercetin because of its high biological activity (Jafar and Nazhad, 2011). It was found that the complex [VO (Quer)2EtOH] n(QuerVO) exerted osteogenic effects because it stimulated type I collagen production and was a weak inhibitory agent upon alkaline phosphatase (ALP) activity (Ferrer et al., 2006). Moreover, QuerVO stimulated phosphokinase in a dose-response manner involved as one of the possible mechanisms for biological effects of the complex. A novel synthesis of the cobalt-quercetin complex was described (Bukhari et al., 2008) and the metal complex was more effective with free radical scavengers than the free flavonoids *in vitro*. Zhou et al. (2001) synthesized eight rare earth metal (III) complexes with quercetin. The antioxidative and antitumor activities of the complexes were tested. The results showed that the suppression ratios of the complexes against the tested tumor cells were superior to quercetin and indicated that the interaction of the complex with DNA was very evident. Tan et al. (2009) found that the quercetin-nickel (II) complex and quercetin-zinc (II) complex can intercalate into stacked base pairs of DNA, which could induce apoptosis of cancer cells. In addition, the complexes which were subjected to biological tests *in vitro* showed significant cytotoxicity against three tumor cell lines (HepG2, SMMC7721 and A549).

Germanium (Ge) is one of the trace elements, which has important physiological activities (Chen and Lin, 2011). Because Ge-132, first synthesized by the Japanese in 1971, showed anticancer activity, organic Ge has been the focus of much attention in drug development (John, 1987). In addition, organic Ge can regulate the immune and hematopoietic function, eliminate free radicals and demonstrate anticancer and anti-inflammatory effects (Levason et al., 2011; Torralvo and Pereira, 2011). The

purpose of the present study was to prepare the complex of quercetin based on its complexation reaction with Ge at alkaline conditions and its anticancer activity was tested.

Results and discussion

Physicochemical properties of the complex

Quercetin-Ge (IV) which was synthesized in the laboratory is stable at room temperature. It is soluble in MeOH, EtOH and DMSO, slightly soluble in Me_2CO and ethyl acetate but insoluble in benzene, toluene and CCl_4.

Synthesis and characterization

Job's method (continual variation method) was applied to validate the stoichiometric composition of the chelate. The absorbance band of ligand quercetin decreased at 375 nm and a new characteristic band of complex was observed at 398 nm. The absorbance plots at 398 nm against the molar fraction of quercetin (X) had a maximum absorbance at XL = 0.66 (Figure 1), confirming that the stoichiometric ratio for the quercetin-Ge (IV) complex was 1:2.

Elemental analysis of the complex

Calculations of percentages of carbon and hydrogen of quercetin-Ge were performed using a PE 2400CHN elemental analyzer (Perkin-Elmer, USA). Elemental analysis calculated (%) for $[C_{30}H_{18}O_{14}Ge{\cdot}2H_2O]{\cdot}2H_2O$ (746.5 g/mol): C 48.2, H 3.6, Ge 9.7, found: C49.1, H 3.2, Ge 9.2.

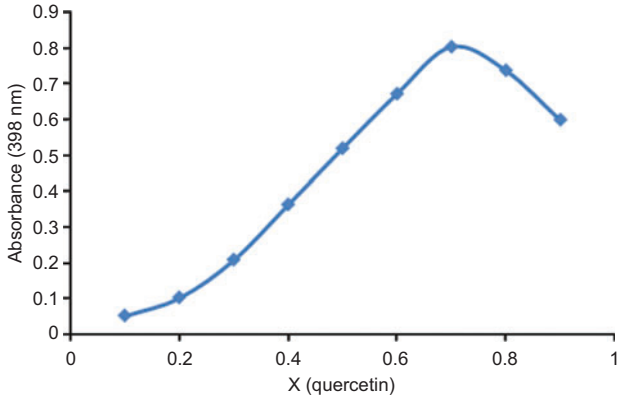

Figure 1 Job's plot for the Ge(IV)-quercetin complex.

UV-vis spectroscopic study of the complex

Flavonoids are a family of polyphenolic compounds whose parent structure is 2-phenyl-benzo-γ-pyrones. Moreover, flavonoids have two absorption peaks at 300–400 nm which belong to cinnamoyl derivatives (B ring) and 240–280 nm which belong to benzoyl derivatives (A ring) separately (Cornard and Merlin, 2002). The UV-vis spectrum of the free quercetin and quercetin-Ge complex in MeOH is presented in Figure 2. Quercetin, like most flavones, exhibits two major absorption bands in the UV-vis region, namely 375 nm (band I) representing B ring absorption (cinnamoyl system) and 255 nm (band II) associated with the absorption involving the A ring benzoyl system as presented in Figure 3 (Ren et al., 2008; Bukhari et al., 2009).

The spectra are related to the π-π* transitions within the aromatic ring of the ligand molecules (Leopoldini et al., 2006). In comparison with quercetin absorption spectra, the band of the complex was shifted to the long wavelength region as presented in Figure 2. The isosbestic point which was characteristic of the formation of a complex was observed at 398 nm. The bathochromic shift could be explained by the extension of the conjugated system with the complexation. Three available sites were identified on quercetin molecules to form metal complexes: 3-hydroxy-4-keto group, 5-hydroxy-4-keto group and ortho-dihydroxy group of B ring (Sun et al., 2008). In this experiment, 3′,4′-hydroxy was dissociated in alkaline condition. After chelating Ge (IV) ion, each of the two bands had a shift (from 375 to 398 nm for band I and from 255 to 259 nm for band II). The shift of band II was obviously higher than that of band I, indicating that the chelation had more conjugative effects on the benzoyl system of A ring than on the cinnamoyl system of B ring. Accordingly, it could be

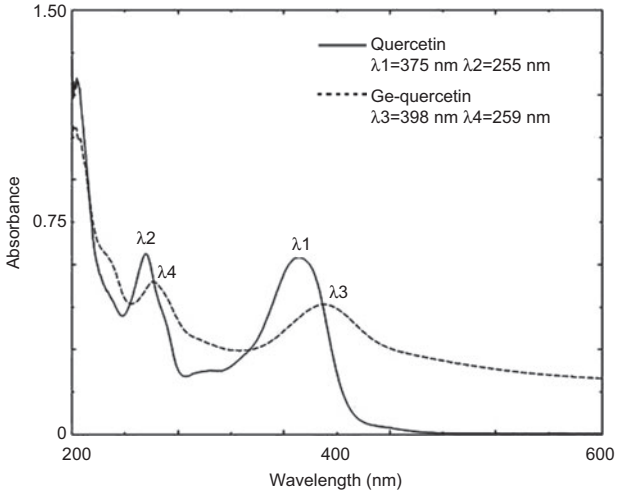

Figure 2 UV spectrum of quercetin and quercetin-Ge (IV).

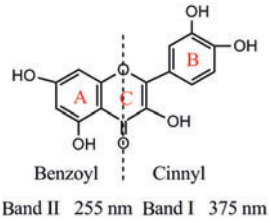

Figure 3 Structure of quercetin.

concluded that the deprotonated 3-OH would coordinate Ge (IV) ion to form the quercetin-Ge (IV) complex.

IR study of the complex

The IR spectroscopies of the ligand quercetin and its complex with Ge (IV) ion are analyzed and data are summarized in Table 1. The spectra present evidence for coordination between metal ions and flavonoid molecules (Ana and Daniel, 2004).

- The ν(O–H) frequencies which appeared as broad bands may be assigned for the presence of water, which was also supported by thermal analysis (Zhou and Sadik, 2008).
- The characteristic stretching ν(C=O) mode of the quercetin occurred at 1663 cm^{-1},whereas due to the formation of Ge (IV) complex, the band appeared at 1654 cm^{-1}. It could be assigned that the Ge (IV) coordination occurred through the carbonyl oxygen atom and the 5-OH or 3-OH group of the quercetin.
- The ν(C=C) of C ring changed slightly upon complexation (1 cm^{-1} from 1610 cm^{-1} of quercetin to 1609 cm^{-1} of quercetin-Ge complex), indicating that the C=C double bond was not involved in chelation.
- The shift of ν(C–O–C) vibration frequency was much slighter(2 cm^{-1} from 1262 cm^{-1} to 1264 cm^{-1}) than that of ν(C=O) vibration frequency, indicating that the O atom of C4=O carbonyl group, but not the O atom in ring C, was involved in chelation.
- The presence of ν(Ge–O) stretching vibration at 636 cm^{-1} indicated the formation of metal complex, whereas quercetin exhibited no such band (Zhou et al., 2007).

^{1}H NMR study

^{1}H NMR data for the Ge-complex, as well as that for free quercetin in DMSO, are presented in Table 2. Results showed the absence of hydrogen of the 3-OH group due to complexation. It also proved that the oxygen atom of the 3-OH group of the ligand was coordinated with Ge (IV) ion. It may be concluded from the results that in the complex, quercetin was chelated with Ge(IV) via 4 C=O and 3 C–O.

Thermal study of the complex

Thermal methods of analysis provided interesting ways for the investigation of metal complexes. Heating of material caused occurrence of chemical and physicochemical transformations, which were accompanied by the absorption or liberation of heat (Lekka et al., 2009). The stability and structure of the complex could be determined by the thermogravimetric analysis (Ohnishi et al., 2008). Thermogravimetric analysis was carried out for the quercetin-Ge(IV) complex under the flow of air. The characteristic thermal changes that occurred with heating rate of 10°C/min from room temperature to 215°C are presented in Figure 4.

It was found that weight loss was 5.08% below 100°C which may be attributed to the liberation of two moles of hydrated water molecules. When the complex was heated to 215°C, weight loss was 5.46%, which may be due to loss of the two coordinated water molecules. Results indicated that the quercetin-Ge complex contains two coordinated water molecules and one crystal water molecule.

The structure of the complex

Characterization of the structure of the complex utilizing elemental analysis, UV, IR and thermogravimetric analysis identified quercetin-Ge (IV) as presented in Figure 5.

Compound	ν_{O-H}	$\nu_{C=O}$	$\nu_{C2=C3}$	$\nu_{C=C(B)}$	$\nu_{C=C(A)}$	δ_{C3-OH}	ν_{C-O-C}	ν_{O-Ge}
Quercetin[a]	(3408)b,m	(1663)m	(1610)s	(1560)m	(1521)s	(1382)s	(1262)m	–
Quercetin-Ge[a]	(3388)b,m	(1654)m	(1609)s	(1484)m	(1434)s	(1400)s	(1264)m	(636)s

Table 1 IR spectra data (cm^{-1}) of quercetin and the quercetin-Ge(IV) complex.
[a]b, broad bands; m, medium; s, strong; w, weak.

	Chemical shift (δ, ppm)									
	5-OH	7-OH	3-OH	4′-OH	3′-OH	2′-H	6′-H	5′-H	8-H	6-H
Quercetin	12.48	10.77	9.58	9.39	9.31	7.64	7.53	6.88	6.39	6.13
Quercetin-Ge	12.72	10.71	–	8.98	8.48	7.38	7.18	6.61	6.38	6.15

Table 2 ^{1}H NMR data of quercetin and the quercetin-Ge (IV) complex.

Inhibition of cells proliferation by quercetin-Ge (IV)

The quercetin-Ge complex was tested for antitumor activity using the MTT assay at concentrations of 10 μM and 100 μM against four human cancer cell lines, namely Hela (human uterine cervix cancer cell line), SPC-A-1 (human lung adenocarcinoma cell line), EC9706 (human esophageal cancer cell line), PC-3 (human prostate cancer cell line). After being co-cultured with the complex for 72 h, the morphological changes of the cells were observed and recorded under the reverse microscope.

The quercetin-Ge (IV) complex tested had a significant inhibitory effect on the growth of four human cancer cells (Table 3). After testing the four human cancer cells with the quercetin-Ge (IV) complex, significant morphological alterations indicative of apoptosis such as cell rounding and shrinkage, retraction from neighboring cells were observed in the cells, whereas the cells in the control group grew well (Figure 6). It was shown from the antitumor experiment *in vitro* that 100 μM of quercetin-Ge could inhibit proliferation of PC-3, EC9706 and Hela cells ($p < 0.01$), especially of SPC-A-1 cells, the inhibitory rate was 47.24%.

The possible mechanism of its anticancer and apoptosis-inducing activities may be due to favorable planarity of the quercetin. The complex can intercalate into DNA, which could induce oxidative DNA damage (Tan et al., 2007). However, the real apoptosis mechanism needs to be confirmed through further detailed research.

Conclusion

A new complex of Ge (IV) with quercetin was prepared under laboratory conditions and characterized utilizing elemental analysis, thermal analysis, UV-vis and IR spectrometric techniques. Spectroscopic data showed 3-hydroxyl-4-keto and not 5-hydroxyl-4-keto as a coordination site. The antitumor activity of the complex was determined by the MTT assay. Results indicated that the complex significantly inhibited proliferation of four tumor cell lines (PC-3, Hela, EC9706 and SPC-A-1) *in vitro*. The possible mechanism of its antitumor activity may be attributed to DNA binding to the complex, but more studies are needed to prove our finding. The complex, as an antitumor drug, may prove to be of application in target-based cancer therapy in the future.

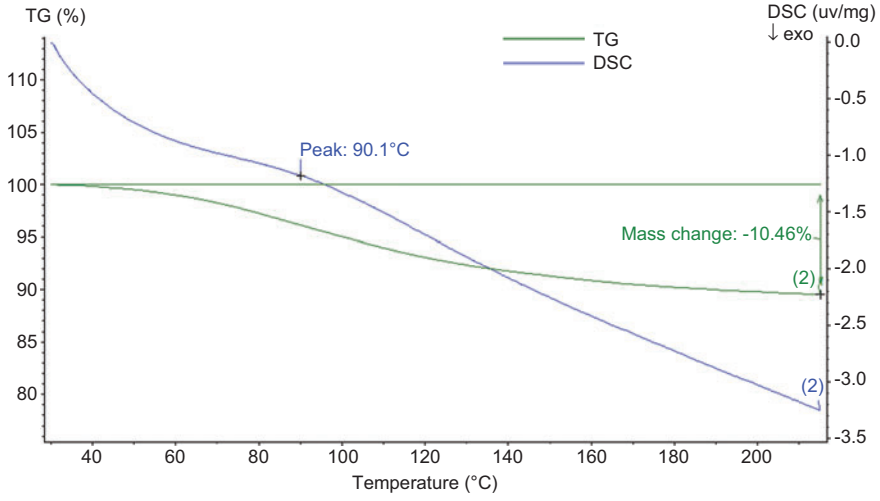

Figure 4 Thermogravimetric analysis of the quercetin-Ge complex.

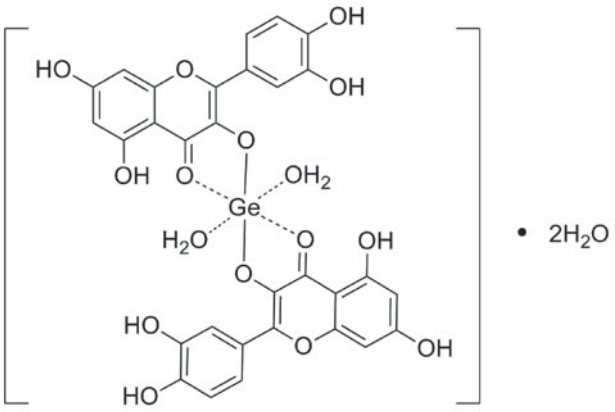

Figure 5 Structure of the quercetin-Ge complex.

Experimental section

Materials and reagents

Quercetin and Ge dioxide were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All reagents were weighed with an accuracy of ±0.0001 g.

Four tumor cell lines (PC-3, EC9706, Hela and SPC-A-1) were purchased from the Institute of Biochemistry and Cell Biology of Shanghai (China). Cells were maintained in RPMI-1640 medium (HyClone, Beijing, China), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 37°C with 5% CO_2 and 95% air atmosphere in a humidified incubator. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Sigma Chemical Co.). All other reagent and solvents were of analytical reagent grade.

Instrumentation

The UV spectrum was recorded on a UV-2550 ultraviolet-visible spectrophotometer (Shimadzu Corporation, KBr). Infrared spectra were recorded using KBr discs on a FTS-3000 spectrometer. Ultimate analysis was performed by a PE 2400CHN elemental analyzer (Perkin-Elmer, USA). Thermogravimetric analysis of the complex was performed by the Thermal analyzer STA 409 PC/PG (Netzsch, Germany), from room temperature to 215°C at a heating rate of 10°C/min. ^{1}H NMR was performed by a AVANCE 400 MHz nuclear magnetic resonance spectrometer (Bruker, Germany).

Synthesis of the quercetin-Ge (IV) complex

Quercetin (302 mg, 1 mmol) was dissolved in methanol (25 ml) with a 100-ml three-neck bottle. Ge dioxide (108.5 mg, 1 mmol) dissolved in methanol (25 ml) was added, then its pH value was adjusted to 8.5 with 40% sodium hydroxide. The reaction mixture was refluxed for 6 h and monitored by TLC. Then the solvent was removed under reduced pressure to obtain the crude product. The residue was separated by polyamide column chromatography (elution with water at first, then gradient elution with methanol). The particular bands were collected, which contained the products. The collection was evaporated under reduced pressure and recrystallized to obtain the orange solid.

Stoichiometric ratio of the metal and ligand in the complex

Job's method (continual variation method) was used to determine the stoichiometric ratio between the quercetin and the metal ion for their complexation in phosphate buffer saline, by mixing solutions of both components of equimolar concentration (1×10^{-3} M) in different ratios varying from 1:9 to 9:1. Then the absorbance was measured at 398 nm.

Antitumor activity of the quercetin-Ge complex by the MTT method

Cells at a density of 6×10^3 cells/well were plated in media containing 10% fetal bovine serum in 96-well plates and allowed to attach overnight. Different final concentrations of the quercetin-Ge (IV) complex (10 μM, 100 μM) were added into the experimental group. Culture medium was added into the control group. Each dose was tested in at least six replicate wells. The cell viability was determined by the conventional MTT reduction assay. The MTT is a substrate for intracellular and plasma membrane oxidoreductase, and its reduction is an indication of cellular metabolic activity. MTT is reduced by mitochondrial enzyme to form formazan, the amount of which is directly related to the number of living cells. After 72 h of incubation, cells were treated with the MTT solution (20 μl/well, final concentration: 0.5 mg/ml) for 4 h. The medium was removed and DMSO (150 μl) was added to each well. The formazan dye crystals were solubilized for 20 min and absorbance measured at 570 nm with a microplate reader. The inhibition rate of the cell growth was calculated as follows: GI (growth inhibiting) = 1 - (OD value of the drug group/OD value of the control group) × 100%.

Compound	Concentration (μm)	72 h Inhibitory rate (%)			
		EC9706	PC-3	Hela	SPC-A-1
Quercetin-Ge (IV)	10	8.67 ± 5.16	7.28 ± 4.18	3.86 ± 3.85	5.04 ± 0.76
	100	35.12 ± 3.80*	29.72 ± 2.84**	33.72 ± 6.07*	47.24 ± 2.09*

Table 3 Inhibitory rate on tumor cells treated with different samples by the MTT assay (n = 6, mean ± SE inhibitory rate/%).
*$p < 0.001$, ** $p < 0.01$ compared with the control group.

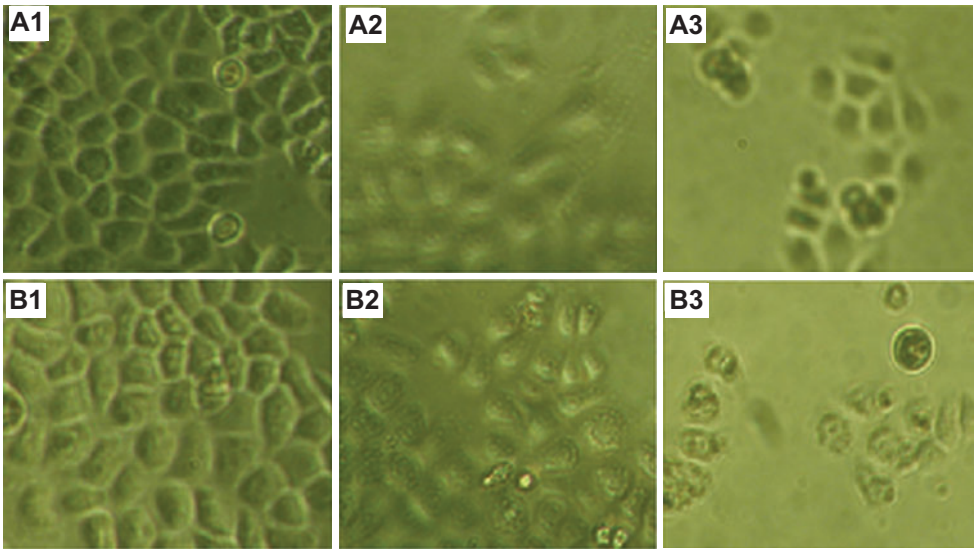

Figure 6 Morphological alterations of cells after treating with the quercetin-Ge complex. Original magnification: 200×. (A) SPC-A-1; (B) EC9706.

Statistical analysis

All data are presented as mean ± SE. The significance of intergroup differences was evaluated by one-way analyses of variance. Differences between groups were considered significant at $p < 0.05$.

Acknowledgments: This work was financially supported by the innovative experiment project for the undergraduate students of Zhengzhou University.

Received June 7, 2012; accepted July 11, 2012; previously published online August 18, 2012

References

Agnes, W. B.; Guido, R. M. M. H.; Aalt, B. Health effects of quercetin: from antioxidant to nutraceutical. *Eur. J. Pharmacol.* **2008**, *585*, 325–337.

Ana, M. M. W.; Daniel, G. M. CHIH-DFT determination of the molecular structure, infrared and ultraviolet spectra of the flavonoid quercetin. *J. Mol. Struct. THEOCHEM* **2004**, *681*, 71–76.

Bravo, A.; Anacona, J. R. Metal complexes of the flavonoid quercetin: antibacterial properties. *Trans. Metal Chem.* **2001**, *26*, 20–23.

Bukhari, S. B.; Memon, S. B.; Tahir, M. M. Synthesis, characterization and investigation of antioxidant activity of cobalt-quercetin complex. *J. Mol. Struct.* **2008**, *892*, 39–46.

Bukhari, S. B.; Memon, S. B.; Tahir, M. M. Synthesis, characterization and antioxidant activity copper-quercetin complex. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2009**, *71*, 1901–1906.

Chen, T. J.; Lin, C. H. Germanium: environmental pollution and health effects. *Encycl. Environ. Health* **2011**, *24*, 927–933.

Chen, C.; Zhou, J.; Ji, C. Y. Quercetin: a potential drug to reverse multidrug resistance. *Life Sci.* **2010**, *87*, 333–338.

Cornard, J. P.; Merlin, J. C. Spectroscopic and structural study of complexes of quercetin with Al (III). *J. Inorg. Biochem.* **2002**, *92*, 19–27.

Erlund, I. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutr. Res.* **2004**, *24*, 851–874.

Ferrer, E.G.; María, V. S.; María, J. C. Synthesis, characterization, antitumoral and osteogenic activities of quercetin vanadyl (IV) complexes. *J. Biol. Inorg. Chem.* **2006**, *11*, 791–801.

Jafar, E.; Nazhad, D. Molecular aspects on the interaction of quercetin and its metal complexes with DNA. *Int. J. Biol. Macromol.* **2011**, *48*, 227–233.

John, S. T. Germapharmaca: some recent studies on biologically active organogermanium compounds. *Appl. Organomet. Chem.* **1987**, *1*, 227.

Lekka, C. H.; Ren, J.; Meng, S. Structural, electronic, and optical properties of representative Cu-flavonoid complexes. *J. Phys. Chem. B* **2009**, *113*, 6478–6483.

Leopoldini, M.; Russo, N.; Chiodo, S. Iron chelation by the powerful antioxidant flavonoid quercetin. *J. Agric. Food Chem.* **2006**, *54*, 6343–6351.

Levason, W.; Reid, G.; Zhang, W. J. Coordination complexes of silicon and germanium halides with neutral ligands. *Coord. Chem. Rev.* **2011**, *255*, 1319–1322.

Moskaug, J. O.; Carlsen, H.; Myhrstad, M.; Blomhoff, R. Molecular imaging of the biological effects of quercetin and quercetin-rich foods. *Mech. Ageing Dev.* **2004**, *125*, 315–324.

Ohnishi, H.; Iwanaga, S.; Kawazoe, K. Effect of iron-quercetin complex on reduction of nitrite in vitro and in vivo systems. *J. Agric. Food Chem.* **2008**, *56*, 10092–10098.

Ren, J.; Meng, S.; Lekka, C. E.; Kaxiras, E. Complexation of flavonoids with iron: structure and optical signatures. *J. Phys. Chem. B* **2008**, *112*, 1845–1850.

Sun, S. F.; Chen, W. J.; Cao, W. Research on the chelation between quercetin and Cr (III) ion by density functional theory (DFT) method. *J. Mol. Struct. THEOCHEM* **2008**, *860*, 40–44.

Tan, J.; Wang, B. C.; Zhu, L. C. Hydrolytic cleavage of DNA by quercetin manganese (II) complexes. *Coll. Surf. B Biointerf.* **2007**, *55*, 149–152.

Tan, J.; Wang, B. C.; Zhu, L. C. DNA binding, cytotoxicity, apoptotic inducing activity, and molecular modeling study of quercetin zinc(II) complex. *Bioorg. Med. Chem.* **2009**, *17*, 614–620.

Torralvo, F. A.; Pereira, C. F. Recovery of germanium from real fly ash leachates by ion-exchange extraction. *Miner. Eng.* **2011**, *24*, 35–41.

Trevor, M. K.; Kathryn, E. K. The effect of quercetin, a widely distributed flavones in food and drink, on cytosolic aldehyde dehydrogenase: a comparison with the effect of diethylstilboestrol. *Biochim. Biophys. Acta* **2000**, *1481*, 247–254.

Zhou, A. L.; Sadik, O. A. Comparative analysis of quercetin oxidation by electrochemical, enzymatic, autoxidation, and free radical generation techniques: a mechanistic study. *J. Agric. Food Chem.* **2008**, *56*, 12081–12091.

Zhou, J.; Wang, L. F.; Wang, J. Y. Antioxidative and anti-tumour activities of solid quercetin metal (II) complexes. *Trans. Metal Chem.* **2001**, *26*, 57–63.

Zhou, A. L.; Kikandi, S.; Sadik, O. A. Electrochemical degradation of quercetin: isolation and structural elucidation of the degradation products. *Electrochem. Commun.* **2007**, *9*, 2246–2255.