

The roles of phytochemicals in red wine as a protective agent against alcohol damage

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Abstract

The study presents how phytochemicals in red wine are used to fight against oxidative stress in humans. In order to determine the antioxidant activity of the phytochemicals, two cell lines human hepatoma HepG2 and human astrocytoma 1321N1 was investigated. The cells were pre-treated with different concentrations of antioxidants (quercetin, gallic acid and epicatechin) followed by treatment with acetaldehyde and acrolein to induce toxicity after 24 hours and cell viability was measured by MTT assay. The result shows that the three antioxidants have good protection on the cells but, this protection is dosage dependent. At higher concentration of antioxidant and toxicity inducing compound (acetylaldehyde and acrolein), the oxidative stress on both HepG2 and human astrocytoma cell line 1321N1 noticeably increased. The research concludes that aside the fact that all the antioxidants are good protective agents, quercetin and epicatechin at the same concentration protects most cells in HepG2 and human astrocytoma cell line 1321N1 respectively while moderate consumption of alcohol or red wine is strongly recommended.

Keywords

Red wine
alcohol
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oxidative stress
antioxidants

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Introduction

Red wine consists of polyphenolic antioxidants which are generally known as the hepatoprotective as well as cardioprotective agents (Ruf *et al.*, 1995). During the last decade, it has been found that, if alcohol is consumed in moderate amount i.e. 10-25 g/day, there is decrease in mortality rate due to cardiovascular disease than those who drink heavily (Marmot *et al.*, 1981). Brain, amongst the organ system responds in a number of ways to alcohol. The brain is damaged due to the formation of free radicals during the alcohol metabolism (Heinz *et al.*, 2003).

Excessive alcohol consumption leads to a range of economic, medical and social consequences (Heinz *et al.*, 2003). Serious illness and disease like cirrhosis, alcoholic fatty liver, cardiovascular diseases are all a result of long term use of alcohol (Ponnappa *et al.*, 2000). In most of the pathways leading to the damage caused by alcohol, oxidative stress is one factor that plays the central role (Ponnappa *et al.*, 2000; Heinz *et al.*, 2003). Reactive oxygen species are generated during metabolic processes in the cells and leads to an imbalance between antioxidants and pro-oxidants due to destruction of antioxidant defence systems

known as "Oxidative Stress" (Schlorff *et al.*, 1999).

Studies has been done to show that many neurological disorders like Alzheimer's disease and age- related brain degeneration can be prevented by wine (Bastianetto *et al.*, 2000). Studies have also demonstrated to date that the intake of phytochemicals can have mechanisms including scavenging of oxidative agents, hormone metabolism, immune system stimulation and antiviral as well as antibacterial effects (Dragsted *et al.*, 1993). Particularly, red wine is found to have more cardio-protective effect than that in other alcohol beverages (Viera *et al.*, 1998). This can however be related to the presence of phenolic compounds playing significant roles in cardio protections (Soleas *et al.*, 1997a; Viera *et al.*, 1998).

The compounds having health benefits in wine comes under a group known as polyphenolics (i.e. a number of hydroxyl groups present in the compounds) (Natsume *et al.*, 2000; Kinjo *et al.*, 2006). These phenolics in turn include two subgroups namely flavonoids and Non-flavonoids (Kinjo *et al.*, 2006). Some of the major flavonoids include quercetin, catechin, anthocyanins and flavonols (Howard and Kritchevsky, 1997). The major compounds in wine

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under Non-flavonoids include stilbenes, resveratrol with health benefits (and hydroxycinnamates (Soleas *et al.*, 1997b). (-)-Epicatechin is a flavonoid that is generally present in red wine, green tea, cocoa products etc. (Natsume *et al.*, 2000). It has been reported that epicatechin can prevent free radical formation and cell death and thereby protects the cells from oxidative stress showing cyto-protective activity in hepatic cells (Kinjo *et al.*, 2006). It has also been reported in another study on the time course regulation of the survival by epicatechin, that pre-treatment by 10 $\mu\text{mol/L}$ concentration of epicatechin leads to the protection of HepG2 cells induced with t-BOOH (Serrano *et al.*, 2009). So, this paper describes the roles of phytochemicals present in red wine which can prevent oxidative stress through the consumption of alcohol.

Materials and Methods

Materials

Human transformed hepatic HepG2 cells, 1321N1 cell line, Dulbecco's modified Eagle's medium (DMEM), 1% Fetal Bovine Serum, 10% solution of Penicillin-Streptomycin, 1% non essential amino acid solution, Quercetin, Epicatechin, Gallic acid, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5- diphenyl-2H-tetrazolium bromide (MTT), phosphate-buffer saline – PBS, Ca^{2+} and Mg^{2+} free, trypsin to detach the cells from the flask, acetaldehyde, acrolein.

Cell culture

In the presence of 500 ml DMEM, routinely HepG2 cells were grown in the flask in a monolayer culture. Also, 10% Fetal Bovine serum, 1% sodium pyruvate, 1% non essential amino acids and 1% penicillin-streptomycin solution were added to the medium. Cells were kept in the incubator and grown in 5% CO_2 (humidified atmosphere) at 37°C. Medium was changed twice a week and the sub-confluent cells (80%) were then treated with trypsin to harvest them from the flask. Cells were re-suspended in the media and about 1×10^3 cells were seeded per well in 96- well plates for cell viability and cytotoxicity assay. Cells were counted by hemacytometer (Hausser Scientific 3200, USA).

Cell treatment

Gallic acid, quercetin and epicatechin were diluted in Dimethyl sulfoxide (DMSO). They were then mixed with the medium and DMSO's final concentration was not greater than 0.1%. The cells in the plates after incubation of about 24 hours were then pre-treated with 5 μM , 10 μM and 30 μM of

Gallic acid (Bachrach and Wang, 2002), 0.1 μM , 1 μM and 10 μM of quercetin (Ali'a *et al.*, 2006) and 10 μM , 25 μM and 50 μM of epicatechin (Serrano *et al.*, 2009). Again, after 24 hours, the compounds and media were taken out. HepG2 cells were treated with 100 μl of 10 μM , 100 μM , 1 mM and 10 mM of acetaldehyde and 100 μM and 1 mM of acrolein. Astrocytoma 1321N1 cells were treated with 10 mM and 100 mM of acetaldehyde and 1 mM and 100 mM of acrolein to induce toxicity in the cells.

Cell viability

MTT assay was used to take out the cell viability. After the treatment of the cells with compounds and acetaldehyde, 20 μl of MTT solution (1.2 mg/ml) was added to the wells. After some time when formazon with purple colour was formed by reduction of MTT by mitochondria, medium with MTT solution was taken out and 100 μl of Dimethyl sulphoxide was added to the wells so that the formazon is resolved (Wu *et al.*, 2007). Microplate reader (1014-Dynex Dias Microplate Reader, Canada) was used to record the absorbance at 560 nm.

Statistical analysis

Values were expressed as mean \pm S.D. of three samples per condition. Analysis and evaluation of the results were done by one way Analysis of Variance (ANOVA) using Minitab 15 software considering $p < 0.05$ as significant.

Results and Discussion

In this study, the human hepatoma HepG2 cell was pre-treated with quercetin prior to induction of oxidative stress by acetaldehyde and acrolein for 24 hours. Cell viability evaluated by MTT assay revealed that the cells treated with quercetin showed partial or complete protection against oxidative stress, especially at higher concentrations of quercetin (10 μM) (Figures 1 and 2). Quercetin is considered to protect the cells due to its high *in vitro* antioxidant and anti-proliferative activity against oxidative injury produced due to ROS (reactive oxygen species) generation. Quercetin acts as free radical scavenger as well as exhibit peroxy radical scavenging activity (Dok-Go *et al.*, 2003). Similar effect was mentioned by Ali'a *et al.* (2006) in which they used t-BOOH to induce oxidative insult in HepG2 cells instead of Acetaldehyde or acrolein. They also showed the protective effect of quercetin against oxidative stress.

In the case of 1321N1 cells, quercetin was able to protect the cells only against toxicity induced by 10 mM acrolein (Figures 3 and 4). There was very

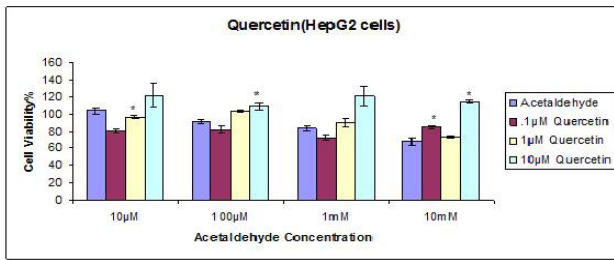


Figure 1. Protective effect of Quercetin at three concentrations (0.1 µM, 1 µM and 10 µM) on cell viability against oxidative injury induced by acetaldehyde. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acetaldehyde treated cells, $p < 0.05$.

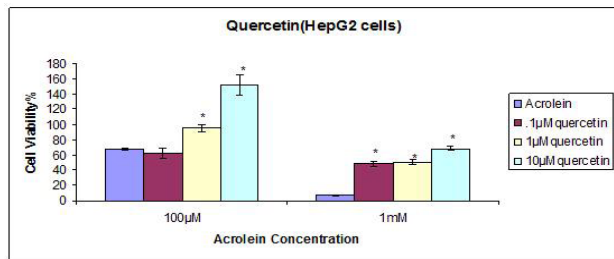


Figure 2. Protective effect of quercetin at three concentrations (0.1 µM, 1 µM and 10µM) on cell viability against oxidative injury induced by acrolein. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acrolein treated cells, $p < 0.05$.

little or no protection seen in other treated cells (i.e. lower concentrations). This may be as a result of the fact that very little oxidative stress was induced by acetaldehyde and acrolein. The disturbances induced in the liver by ethanol were mainly due to generation of oxidative stress as well as the generation of ROS (Dupont *et al.*, 2000). So, the inability to eliminate ROS by cells as a result of their higher levels or reduction in the normal levels of antioxidants due to toxic insults like alcohol leads to the oxidative stress which in turn cause damage to DNA, membranes, as well as causes cell death by release of factors inducing apoptosis (Bredensen, 1996). Therefore, quercetin from red wine consumed at suitable level may contribute to protection against diseases caused by the production of excess ROS. This shows that the higher the oxidative damage produced by the toxicity inducing compound, the higher protective effect shown by the protective compounds.

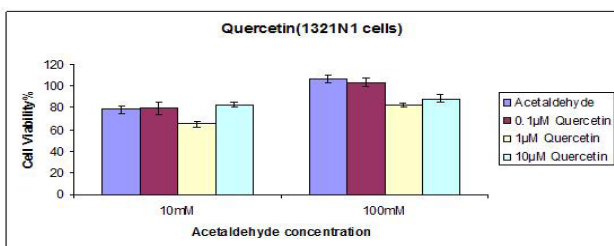


Figure 3. Protective effect of quercetin at three concentrations (0.1 µM, 1 µM and 10 µM) on cell viability against oxidative injury induced by acetaldehyde. Data is mean + S.D. of three samples per condition.

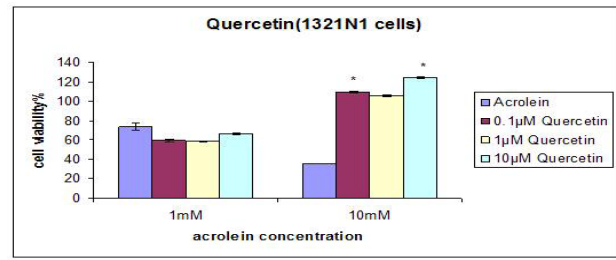


Figure 4. Protective effect of quercetin at three concentrations (0.1 µM, 1 µM and 10 µM) on cell viability against oxidative injury induced by acrolein. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acrolein treated cells, $p < 0.05$.

In various chronic diseases like neurodegenerative disorders and cancer, quercetin has been found to show positive health benefits (Edwin Shackelford *et al.*, 2005). Quercetin has the ability to prevent the oxidation of glutathione (GSH) which helps in the protection of neurotoxicity induced by oxidative stress (Ishige *et al.*, 2001). In a similar experiment, it was found that quercetin as an antioxidant can help protect brain from cytotoxicity induced by H_2O_2 (Heo and Lee, 2004). The results from the assay clearly suggest that if cells are treated with quercetin at higher concentrations, it may prepare the cell's antioxidant defence system in order to fight against oxidative stress.

From the MTT assay results, it can be seen that Gallic acid, especially at higher concentration (30 µM) had protective effect against oxidative injury induced by acetaldehyde (Figure 5) and acrolein (Figure 6) in HepG2 cells. At 10 µM concentration of acetaldehyde in case of HepG2 cells, higher concentration of Gallic acid did not show protective effect. This may be as a result of the fact that no cell damage was induced by acetaldehyde and so Gallic acid at higher concentration showed cytotoxic to the cells (Li *et al.*, 2010). Also, Gallic acid showed a dose dependent increase in protective effect against the cell damage induced by 100 µM and 1 mM concentrations of acetaldehyde and both the concentrations of acrolein in HepG2 cells, i.e. higher display of protective effects at higher concentration of Gallic acid. A similar result was also reported by Li *et al.* (2010) they pre-treated human hepatocytes (HL - 7702 cell line) with Gallic acid prior to treatment of the cells with H_2O_2 and CCl_4 . The reason Gallic acid enhanced cell viability and protect the cells against oxidative stress can be linked to its ability to reduce GSH depletion and decrease in lactate dehydrogenase (LDH) leakage.

In the case of 1321N1 cells, Gallic acid did not show protective effect in cells treated with 100 mM acetaldehyde (Figure 7) and 1 mM acrolein (Figure 8). This may be related to the fact that very little

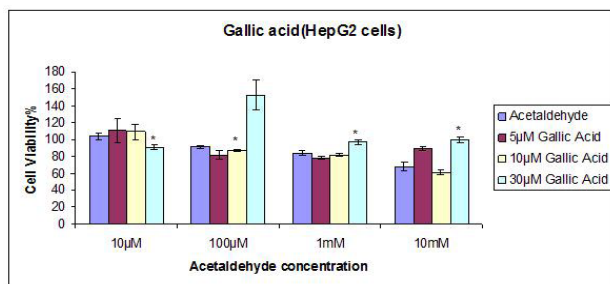


Figure 5. Protective effect of Gallic acid at three concentrations (5 µM, 10 µM and 30 µM) on cell viability against oxidative injury induced by acetaldehyde. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acetaldehyde treated cells, $p < 0.05$.

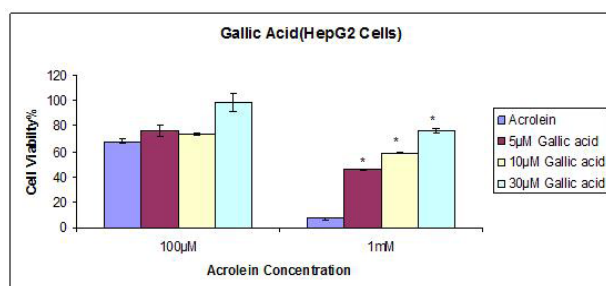


Figure 6. Protective effect of Gallic acid at three concentrations (5 µM, 10 µM and 30 µM) on cell viability against oxidative injury induced by acrolein. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acrolein treated cells, $p < 0.05$.

oxidative stress was induced by both acetaldehyde and acrolein at these concentrations and so in comparison to this, Gallic acid showed very little protective effect. Gallic acid is known to be a strong antioxidant with activities like anticarcinogenic and antimutagenic (Inoue *et al.*, 1994). 4-O-methylgallic acid (4OMGA) derivative of Gallic acid has been found to be its main metabolite reported in humans (Shahrzad and Bitsch, 1998) and it is also available in abundance in red wine. It has been reported that various red wines have a total phenolic content of 1100 to 3165 mg/L, out of which 35 to 70 mg/L is comprised of gallic acid (Burns *et al.*, 2000).

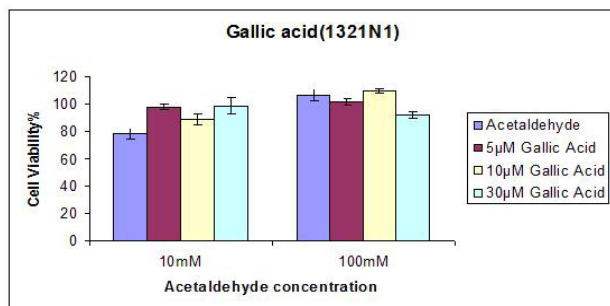


Figure 7. Protective effect of gallic acid at three concentrations (5 µM, 10 µM and 30 µM) on cell viability against oxidative injury induced by acetaldehyde. Data is mean + S.D. of three samples per condition.

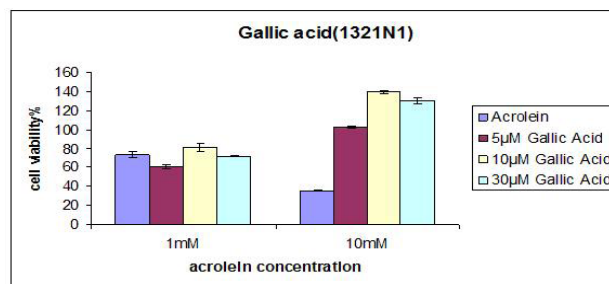


Figure 8. Protective effect of gallic acid at three concentrations (5 µM, 10 µM and 30 µM) on cell viability against oxidative injury induced by acrolein. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acetaldehyde treated cells, $p < 0.05$.

For epicatechin, the result showed that at lower concentrations (10 µM and 25 µM), epicatechin did not show any strong cell proliferation or protection against cell injury at all concentrations of acetaldehyde (Figure 9) and acrolein (Figure 10) in HepG2 cells. At low concentrations, epicatechin was found to induce very little alterations in cell viability and cytotoxicity in HepG2 cells. In conformity to these findings Babich *et al.* (2005) and Galati *et al.*, (2006) reported a minor effect of epicatechin at low concentrations on human hepatic cells HepG2 cells and oral cavity cells. Also, it is known that flavanols at higher concentrations show stronger antioxidant effects, thereby effectively preventing the ROS from damaging the cells (Yamazaki *et al.*, 2008).

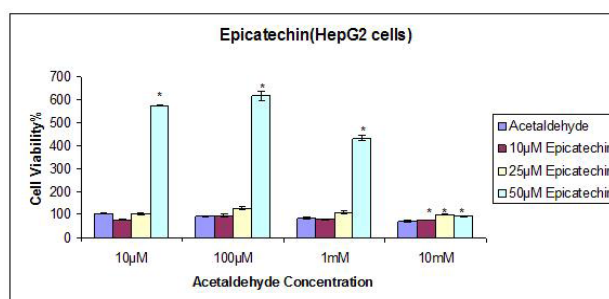


Figure 9. Protective effect of Epicatechin at three concentrations (10 µM, 25 µM and 50 µM) on cell viability against oxidative injury induced by acetaldehyde. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acetaldehyde treated cells, $p < 0.05$.

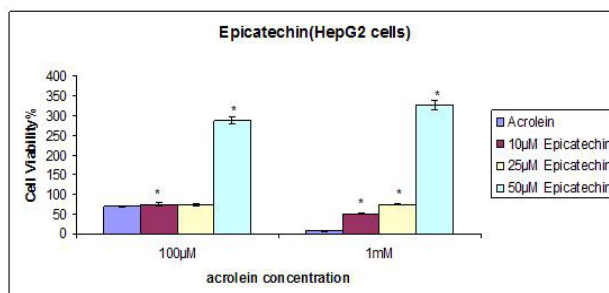


Figure 10. Protective effect of Epicatechin at three concentrations (10 µM, 25 µM and 50 µM) on cell viability against oxidative injury induced by acrolein. Data is mean + S.D. of three samples per condition. Asterisks represent the significance compared with the acrolein treated cells, $p < 0.05$.

At higher concentrations of epicatechin (50 μM), there was a strong protection seen against the oxidative stress induced at all concentrations of acetaldehyde and acrolein in HepG2 cells. Due to the high antioxidant activity and cytoprotective effect, epicatechin at higher concentrations have shown to protect the hepatic cells against oxidative injury by prevention of the formation of free radicals and cell death in the presence of toxicity producing compounds (Roig *et al.*, 2002; Kinjo *et al.*, 2006).

In 1321N1 cells, epicatechin was found to show protective effects against oxidative stress produced by both acetylaldehyde (Figure 11) as well as acrolein (Figure 12) which may be due to superoxide radical scavenging activity of epicatechin. Also, it has been found that after different times of incubation, cells treated with epicatechin alone resulted in a slight decrease or unchanged levels of formation of ROS (Chung *et al.*, 2001; Hernandez *et al.*, 2007). So, results suggest that protective agents present in red wine were able to provide protection to the cells with increase in cell viability mostly at higher concentrations against the toxicity induced.

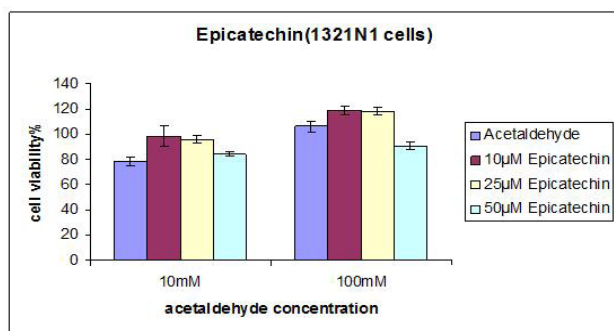


Figure 11. Protective effect of Epicatechin at three concentrations (10 μM , 25 μM and 50 μM) on cell viability against oxidative injury induced by acetaldehyde. Data is mean + S.D. of three samples per condition.

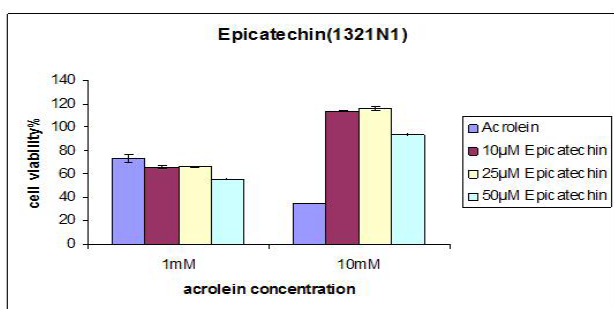


Figure 12. Protective effect of epicatechin at three concentrations (10 μM , 25 μM and 50 μM) on cell viability against oxidative injury induced by acrolein. Data is mean + S.D. of three samples per condition.

In our experiment, the protective influence of the antioxidants is higher with higher concentration of toxicity inducing agent. The result was supported

by the research conducted by Block *et al.* (1992) where a study investigating the relationship between the intakes of phytochemical through fruits and vegetables and cancers in stomach, oral cavity, colon, pancreas etc. It was found that the persons having low intake of vegetables and fruits had an increase risk of cancer than those with high intake of phytochemicals. Therefore, it is very necessary to obtain a balance between the antioxidants and oxidants in order to produce physiological conditions that are optimal in body (Liu and Hotchkiss, 1995).

Conclusion

The human hepatoma HepG2 cells and astrocytoma 1321N1 was successfully assessed for cytoprotection using acetaldehyde and acrolein insults. The findings of the experiment support the hypothesis that after alcohol intake, acetaldehyde and acrolein plays the primary role in inducing toxicity in the cells. It can be said that with increasing concentrations of the toxic insults, there is an increase in the protective effects. The best results were particularly obtained at the highest tested concentrations (50 μM epicatechin, 30 μM Gallic acid, 10 μM quercetin), and with the cells induced with highest concentrations of acetaldehyde and acrolein. The results obtained suggests and also agree with previously reported investigations that phenolic components found in red wine are capable of protecting both the human hepatoma HepG2 cells and astrocytoma 1321N1 cells from oxidative stress induced by toxic compounds. Overall, the protective effect strength of the antioxidants (at 10 μM) for HepG2 cell are in the order quercetin > gallic \approx epicatechin while for 1321N1 cell it is of order epicatechin > gallic > quercetin.

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