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Research Article

The Chemo-preventive Ability of Quercetin and Isorhamnetin on Bromate- induced Cytotoxicity and Oxidative Stress May Involve Modulation of the Inflammatory Response

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ABSTRACT

The protective/preventive effects of quercetin and isorhamnetin on bromate-induced aberrations were studied. Cells were first incubated with either quercetin or isorhamnetin before exposure to bromate. Subsequently, cell viability and production of reactive oxygen species (ROS) were assessed. In the other investigation, U937-derived macrophages were incubated with either flavonoid before exposure to bromate. Subsequently, the production of pro-inflammatory markers and expression of the antioxidant enzymes; superoxide dismutase (SOD) and catalase (CAT) were also investigated. The findings reveal that bromate caused significant cytotoxicity, production of ROS, nitric oxide and cytokines when compared to untreated controls *($p < 0.05$)*. Bromate also reduced the expression of the antioxidant enzymes SOD and CAT. However, chemoprevention was observed when the cells were pre-incubated with either flavonoid which was concentration-dependent *($p < 0.05$)*. Pre-incubation of the cells with the flavonoids also reduced bromate-induced production of reactive oxygen species. The flavonoids also reduced bromate-induced production of nitric oxide and the cytokines in the U937-derived macrophages. The expression of the antioxidant enzymes was also enhanced following the pre-treatment of the macrophages with the flavonoids. Quercetin tends to be more active than isorhamnetin at reducing bromate-induced cytotoxicity and production of ROS. However, isorhamnetin tends to be better at reducing bromate-induced alterations on the production of NO and the pro-inflammatory cytokines but largely not significant. Isorhamnetin was also better at enhancing the expression of antioxidant enzymes than quercetin *($p < 0.05$)*.

Keywords: *Antioxidant, Bromate, Cytotoxicity, Cytokines, Isorhamnetin, Quercetin*

INTRODUCTION

The consumption of plants/plant-derived products for health enhancement has been in existence for decades in some regions. In such areas, plants have been utilized as first line defenses against many ailments. It has been reported that there are less frequent side effects following their use when compared to many synthetic drugs (Okoko and Oruambo, 2008; Awuchi, 2019).

The efficacy of these edible plants as medicinals has largely been ascribed to the possession of phenolic compounds which have been reported to possess significant antibacterial, anti-inflammatory, cardioprotective, anticancer as well as other properties (Tungmunnithum *et al.,*

2018; Karak, 2019; Al-Snafi, 2020). One of these important polyphenols is quercetin, a yellow coloured flavanol widely present in tea, onion, grapes, tomato, orange etc (Almatroodi *et al.,* 2021; Singh *et al.,* 2021). Quercetin (Figure 1) has been reported to possess significant bioactivities both in *in-vivo*, *in-vitro* and *ex-vivo* models thus quite promising as a nutraceutical (Kim and Park, 2018; Xu *et al.,* 2019). Though the daily intake of quercetin in western diet seems to be high, its bioavailability could be low due to extensive metabolism (Lesjak *et al.*, 2018) thus there is need to investigate the bioactivity of some of its metabolites that are synthesized following its metabolism since it is also thought that these derivatives could possess different efficacies (Lesjak *et al.,* 2018; Sharma *et al.,* 2018).

Bromate (potassium bromate) is an oxidizing agent used in bread making as a dough conditioner and water treatment but its ability to cause significant cytotoxicity, oxidative stress and cancer, has made some countries partially or completely ban its use (Wu *et al.,* 2019; Shanmugavel *et al.,* 2020). The aim of this work is to investigate the chemo-preventive effects of quercetin and one of its metabolites, 3-O-methyl quercetin (also known as isorhamnetin, Figure 1) on bromate-induced cytotoxicity, oxidative stress and inflammatory response in promonocytic U937 cells and macrophages.

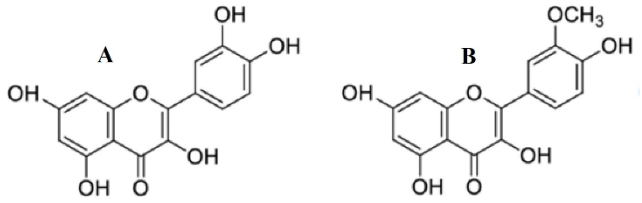

Figure 1. Structure of (A) Quercetin and (B) Isorhamnetin, (Safe et al., 2021).

MATERIALS AND METHODS

Chemicals and reagents

Trypan blue, dimethyl sulfoxide (DMSO), potassium bromate, RPMI-1640 medium (Roswell Park Memorial Institute), phorbol-12-myristate-13-acetate (PMA), *L*-glutamine, penicillin-streptomycin, fetal calf serum (heat inoculated), quercetin, isorhamnetin, 2′,7′-dichlorohydrofluorescein diacetate (DCHF-DA), and 3-(4,5-dimetylthiazol-2-yl),-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemicals (USA). The human macrophage cell line U937 was obtained from the European Collection of Cell Cultures (Salisbury). All antibodies and biotinylated cytokines were products from Pharmingin. All other reagents and chemicals were of analytical grade hence used without further purification. Solutions and buffers were prepared using Milli-Q (18 mΩ/cm) water and stored at room temperature unless otherwise stated.

Cell culture and cytotoxicity

The cell line U937 was cultured in RPMI-1640 medium and maintained at 5 x 10^4 cells/mL as described by Okoko and Oruambo (2009). For toxicity experiment, cells were pre-incubated with either quercetin or isorhamnetin (15 - 50 µM in RPMI) for 24 h before exposure to 4 mM bromate (as potassium bromate). Two hours later, cell viability was determined via the trypan blue dye exclusion assay and MTT reduction assay according to Zhou *et al.* (2006) as modified (Okoko and Ndoni, 2021). The production of reactive oxygen species (ROS) was also assessed via the DCHF – DA assay according to the method of Koga and Meydani (2001) as modified (Okoko, 2020).

Production nitric oxide and pro-inflammatory cytokines

The monocyte cell line U937 (5 x 10^4 cells/mL) was differentiated to macrophages via treatment with phorbol-12-myristate-13-acetate (PMA) as described (Okoko and Oruambo, 2009). Media were aspirated and replaced with or without quercetin or isorhamnetin and incubated for 24 h in a humidified cabinet at 37ºC. Where applicable, cells were incubated with bromate (4 mM) and production of nitric oxide was analysed in each supernatant according to Hwang *et al.* (2002) as modified by Hsieh *et al.* (2007). The production of IL-1, IL-6 and TNF-α was analysed via cytokine ELISA as described by Okoko and Oruambo (2009).

Expression of superoxide dismutase (SOD) and catalase (CAT)

The expression of these antioxidant enzymes was determined by RT-PCR as described (Okoko, 2020). Briefly, total RNA was extracted and purified using a TRIzol kit followed by cDNA synthesis. Sequences specific to SOD and CAT were amplified via RT-PCR using the primer pairs 5′-GACTGAAGGCCTGCATGGATTC-3′/ 5′-CACATCGGCCACACCATCTTTG-3′ (forward and reverse primers for SOD), 5′-CTTCGACCCAAGCAACATGC-3′/5’-GATAATTGGGTCCCAGGCGATG-3’ (forward and reverse primers for CAT) and 5′-GTCGGAGTCAACGGATTTGGTC-3′/5′-CTTCCCGTTCTCAGCCTTGAC-3′ (forward and reverse primers for GAPDH). Results were normalized against GAPDH mRNA (reference housekeeping gene) in each sample.

Statistical analysis

Values are expressed as mean ± SEM from six replicates. Significance of detectable differences was determined by subjecting raw data to analysis of variance followed by Duncan's multiple range tests. Significance was set at $p < 0.05$.

RESULTS

In order to investigate whether the flavonoids could possess inherent toxicity, the cells were first treated with either quercetin or isorhamnetin (25 µM) and incubated for 24 h. It revealed both flavonoids were not toxic to the cells (presented in the bars as 'Quer only' and 'Iso only' in Figure 2). The result further indicated that incubating the cells with bromate resulted in over 70 % cell death as revealed by both trypan

blue dye exclusion and MTT reduction assay (Figure 2). Pre-incubating the cells with either quercetin or isorhamnetin before exposure to bromate showed a gradual increase in cell viability with increasing concentration which was significant in some cases.

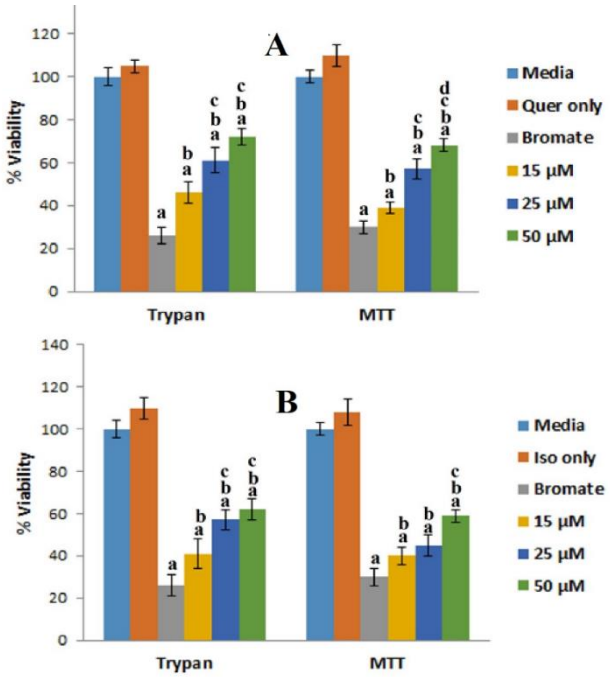

Figure 2. The Effect of (A) Quercetin and (B) Isorhamnetin on Bromate-induced Cytotoxicity Assessed by the Trypan Dye Exclusion (Trypan) and MTT Reduction (MTT) Assays.

Each bar represents mean ± standard error of the mean of six replicates expressed as % viability in comparison to control (control value assigned 100 %). [a]significantly different from Media; [b]significantly different from Bromate; [c]significantly different from 15 µM; [d]significantly different from 25 µM. $p < 0.05$

The effect of the flavonoids on bromate-induced production of ROS as assessed by the DCHF-DA assay is shown in Figure 3. Bromate caused significant production of ROS which was reduced when each of the flavonoids was pre-incubated with the cells. With increasing concentration of flavonoid, there was gradual reduction in ROS production. When the cells were pre-incubated with 25 and 50 µM quercetin, the reductions in ROS production were significantly lower than even the control value ($p < 0.05$). While for pre-incubation with isorhamnetin, only treatment with 50 µM resulted in a similar response.

The abilities of the flavonoids at reducing bromate-induced production of nitric oxide and cytokines are shown in Figures 4 and 5. Bromate caused significant production of nitric oxide in U937-derived macrophages which was reduced when cells were pre-incubated with each flavonoid (Figure 3) which was concentration-dependent ($p < 0.05$). Similarly, treating macrophages with bromate alone significantly increased secretion of the cytokines IL-1, IL-6 and TNF-α which was determined by the cytokine capture assay. Incubating cells with each flavonoid prior to bromate exposure significantly reduced production of the cytokines which was gradual with increasing concentrations (flavonoids).

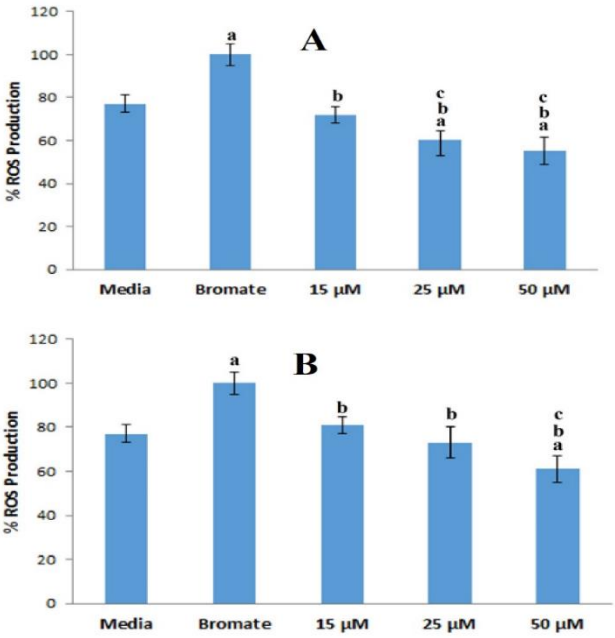

Figure 3. The Effect of (A) Quercetin and (B) Isorhamnetin on Bromate-induced ROS Production.

Each bar represents mean ± standard error of the mean of six replicates expressed as % viability in comparison to Bromate (value assigned 100 %). [a]significantly different from Media; [b]significantly different from Bromate; [c]significantly different from 15 µM; [d]significantly different from 25 µM. $p < 0.05$

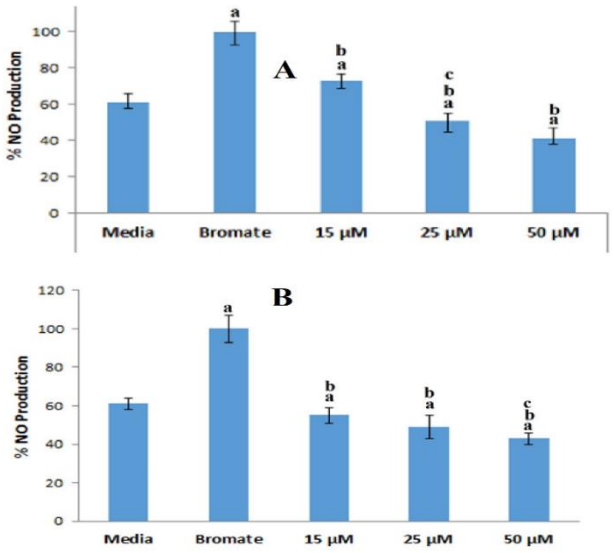

Figure 4. The Effect of (A) Quercetin and (B) Isorhamnetin on Bromate-induced Production of NO.

Each bar represents mean ± standard error of the mean of six replicates expressed as % viability in comparison to Bromate (value assigned 100 %. [a]significantly different from Media; [b]significantly different from Bromate; [c]significantly different from 15 µM; [d]significantly different from 25 µM. $p < 0.05$.

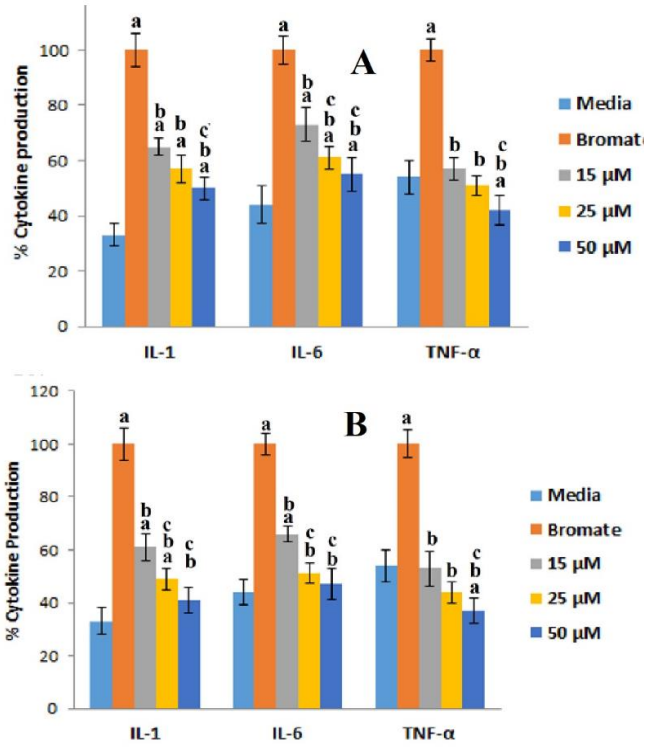

Figure 5. The Effect of (A) Quercetin and (B) Isorhamnetin on Bromate-induced Secretion of Cytokines in U937-derived Macrophages.

Each bar represents mean ± standard error of the mean of six replicates expressed as % viability in comparison to Bromate (value assigned 100 %. [a]significantly different from Media; [b]significantly different from Bromate; [c]significantly different from 15 µM; [d]significantly different from 25 µM. $p < 0.05$.

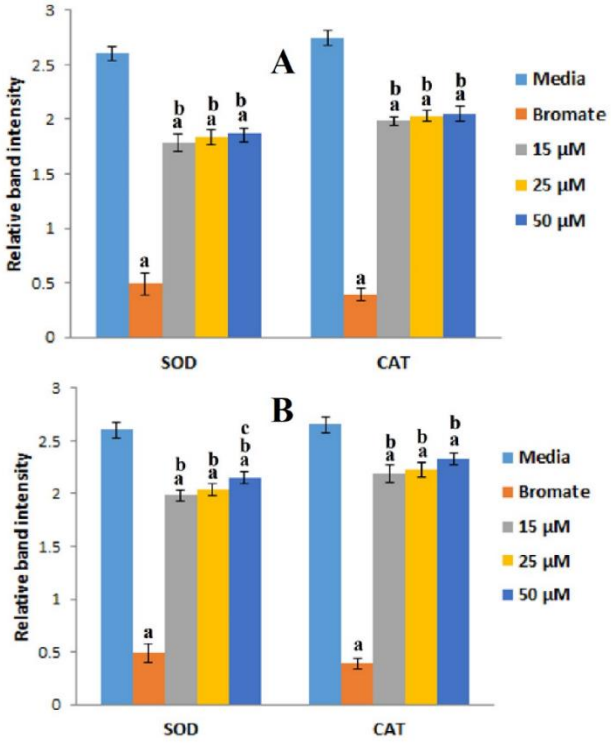

Figure 6. Effect of (A) Quercetin and (B) Isorhamnetin on Bromate-induced Gene Expression.

Each bar represents mean ± standard error of the mean of six replicates [a]significantly different from Media; [b]significantly different from Bromate; [c]significantly different from 15 µM; [d]significantly different from 25 µM. $p < 0.05$

The effect of flavonoids on the expression of the antioxidant SOD and CAT is shown in Figure 6. The RT-PCR data showed that bromate significantly reduced the expression of the antioxidant enzymes. Pre-incubation of the macrophages with flavonoid before exposure to bromate significantly enhanced gene expression closer to control levels ($p < 0.05$). However, the enhancement of gene expression seems not to be concentration-dependent.

Figures 7 and 8 compare the effect of quercetin with isorhamnetin on the bromate-induced alterations. Though the differences are largely not significant, quercetin tends to be more active at reducing bromate-induced cytotoxicity and production of ROS (figure 7). However, isorhamnetin tends to be more active at reducing bromate-induced alterations on the production of NO, the pro-inflammatory cytokines though largely not significant. Isorhamnetin also enhanced the expression of antioxidant enzymes significantly more than quercetin ($p < 0.05$) (Figure 8).

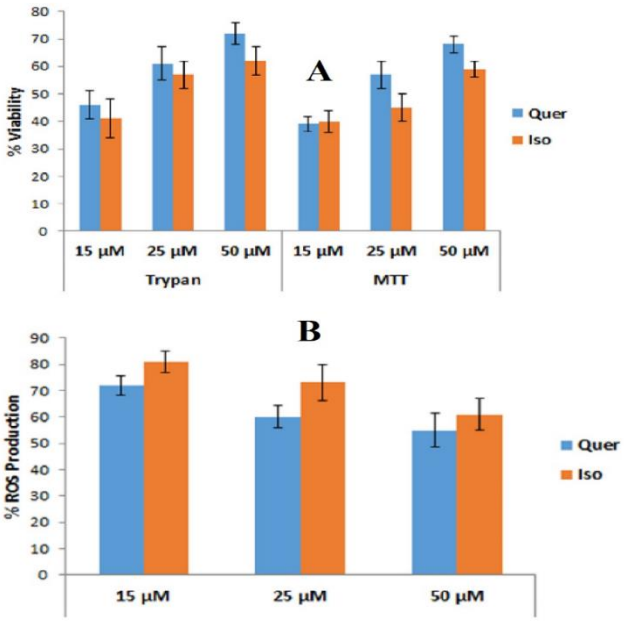

Figure 7. Comparison of Responses Produced by Quercetin and Isorhamnetin on Bromate-induced (A) Cytotoxicity and (B) ROS Production.

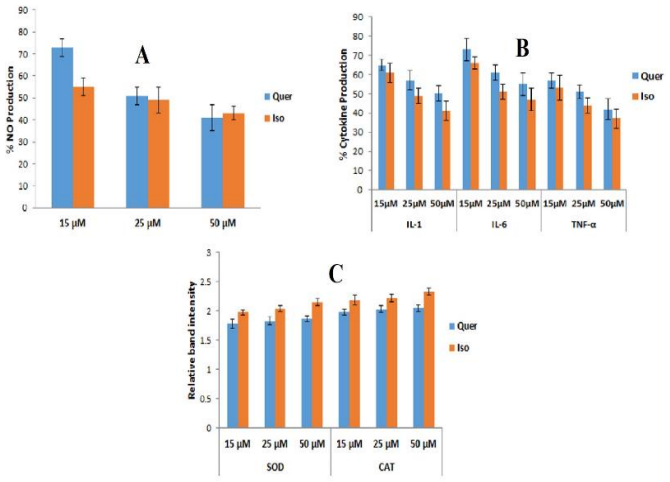

Figure 8. Comparison of Responses Produced by Quercetin and Isorhamnetin on Bromate-induced (A) Production of NO (B) Secretion of Cytokines and (C) Gene Expression

DISCUSSION

Alhough the mechanism via which bromate causes cell death is largely unknown, it is believed that it involves DNA damage mediated by the formation of adducts (Zhang *et al.,* 2010; Memmedor *et al.,* 2020). Cell death could be as a result of oxidative stress and is thought to be mediated by the inhibition of signaling pathways in animal models (Kuo et al., 2018; Memmedor *et al.,* 2020). In the current investigation, bromate-induced cell death and production of reactive oxygen species were significantly prevented by both quercetin and isorhamnetin (figures 2 and 3). It has been suggested that bromate-induced oxidative damage and cell death is a consequence of free radical generation (*Hassan et al.,* 2019). Flavonoids (especially quercetin) protect cells against oxidative damage by direct scavenging and/or stabilization of reactive oxygen species (Mahmoud *et al.,* 2019; Janabi *et al.,* 2020). Quercetin also protects cells by modulating several signal transduction pathways either directly or indirectly (Xu *et al.,* 2019; Yang *et al.,* 2020).

Nitric oxide (NO) is synthesized by macrophages from arginine in a reaction catalyzed by inducible nitric oxide synthase (iNOS). The signal molecule is a pro-inflammatory mediator that plays significant role in the regulation of the immune system (Guzik *et al.,* 2003; Sharma *et al.,* 2007). In response to inflammatory stimulus, NO could be overproduced due to upregulated expression of iNOS and this can cause tissue damage implicated in several disorders (Gliozzi *et al.,* 2009; Cinelli *et al.,* 2020). In a previous study, incubation of U937 macrophages with bromate caused in significant production of NO and pro-inflammatory cytokines (Okoko, 2020) Pro-inflammatory cytokines indirectly cause a delayed but persistent synthesis of NO by stimulating the expression of iNOS (Gliozzi *et al.,* 2009; Albaayit *et al.,* 2019). In this study, incubating the macrophages with the flavonoids before exposure to bromate significantly reduced the production of NO and the cytokines IL-1, IL-6 and TNF-α (Figures 4 and 5). The deregulated production of pro-inflammatory cytokines leads to oxidative stress since NO reacts with superoxide to produce peroxynitrite (a powerful oxidant) which could decompose to the hydroxyl radical-the most reactive of the biologically active free radicals (Macdonald et al., 2003; Elmarakby and Sullivan, 2012). Thus bromate-induced oxidative stress in macrophages could be linked to the elaboration of cytokines. It has been reported that flavonoids inhibit the production of pro-inflammatory cytokines thereby lowering the production of nitric oxide and other reactive oxygen species (Leyva-lopez *et al.,* 2016). The bioactivity of flavonoids (especially quercetin) is thought to be due to the modulation of some signal pathways (Xie *et al.,* 2012; Mansri *et al.,* 2014; Xu *et al.,* 2019; Yang *et al.,* 2020).

Oxidative stress is often caused by the imbalance between oxidants and antioxidants in favour of the oxidants thus we decided to investigate the roles quercetin and isorhamnetin perform on the expression of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). It revealed that the flavonoids significantly inhibited bromate-induced reduction of antioxidant enzyme expression. Thus, the cytoprotective effect of the flavonoids could be linked to the upregulated synthesis of the enzymes.

It has been reported that flavonoids confer direct antioxidant protection of cells while others induce the elaboration of antioxidant enzymes (Tsuji *et al.,* 2013, Chanu et al., 2023). However, some seem to protect cells via both means and chemical substructural fingerprints contribute to their different activities (Alzand and Mohammed, 2012; Tsuji *et al.,* 2013). It has been observed that the hydroxyl group on C-3 is key to the biological activity of quercetin thus methylation at the 3-O position (as it is in isorhamnetin) is expected to significantly modify the bioactivity of quercetin (Ozgen *et al.,* 2016). Lesjak *et al.* (2018) observed that while quercetin possessed higher reducing ability and inhibition of lipid peroxides than isorhamnetin, the reverse is the case on their anti-inflammatory potential and DPPH scavenging activity. However, Karancsi *et al.* (2022) observed that isorhamnetin was better than quercetin at protecting procine intestinal cells from lipopolysaccharide-induced cytotoxicity and production of ROS and IL-6.

In the study, it was observed that while quercetin was better at protecting cells from bromate-induced cytotoxicity and production of ROS in raw U937 cells, the metabolite isorhamnetin was better at reducing the production of NO and pro-inflammatory cytokines. Isorhamnetin was also better at upregulating the synthesis of SOD and catalase.

Even though the observations seem to conflict, the research gave further support to the role chemical modification of quercetin (at the 3-O position) could significantly affect bioactivity.

CONCLUSION

Both quercetin and its *in-vivo* metabolite significantly reduced bromate-induced cytotoxicity and production of ROS. The flavonoids also inhibited bromate-induced secretion of pro-inflammatory cytokines and NO in U937-derived macrophages in addition to upregulation of

antioxidant enzymes. Following intake of quercetin, isorhamnetin is more concentrated than quercetin in plasma which indicates 3-O-methylation increases bioavailability even though it is generally low (Jiang *et al.,* 2019; Kim *et al.,* 2021). Taken together, the findings reveal that the modulation of the inflammatory response by the flavonoids is a consequence of enhanced cell viability and reduction of ROS production.

AUTHORS' CONTRIBUTIONS

TO conceptualized the study including supervision. FOR did the data collection and interpretation. All authors read, corrected and approved the final manuscript.

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Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Not applicable

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