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Research Article

Attenuation of Anxiety/Depression-like Behaviors and Neuroanatomical Changes Following Quercetin and Rutin Exposure in Zebrafish (*Danio rerio*)

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Abstract

The flavonoids quercetin and rutin, present in a variety of fruits, vegetables, and medicinal plants such as St. John's wort (*Hypericum perforatum*), are known to have wide-ranging beneficial effects on human health. The aim of this study was to clarify the mechanisms-of-action underlying changes in anxiety/depression-related behaviors and the neuroanatomical components affected following exposure to quercetin and rutin in zebrafish (*Danio rerio*). Employing behavioral and histological analysis, behavioral improvement similar to that of fluoxetine (Prozac®) treatment and significant upregulation of hippocampal serotonergic and dopaminergic neurons was observed in zebrafish treated with both quercetin and rutin. It is indicated, based on the results of this study, that quercetin and rutin mediate their antidepressant effects through dopaminergic and serotonergic neurotransmission and that dietary quercetin and rutin are promising as antidepressant-like compounds, and possible neuroactive pharmaceutical lead structures. Therefore, further investigations of these and additional secondary plant metabolites for potential antidepressant properties are needed.

Keywords: Quercetin; Rutin; Anxiety; Depression; Zebrafish (*Danio rerio*)

1. Introduction

The focus of this study is on behavioral and neuroanatomical effects of two plant extracts, quercetin and rutin, in zebrafish (*Danio rerio*). Our particular interest is in identifying any potential antidepressant properties these bioactive compounds may exhibit. Zebrafish are becoming a commonly used alternative experimental organism in biomedical research because the physiology and morphology of this species are homologous to humans [1]. Furthermore, the ease of drug exposure with this taxon and subsequent clear behavioral changes makes this organism a powerful model to investigate the neural substrates of anxiety/depression [2]. The World Health Organization estimates that depression and depression-related illnesses will be as costly and debilitating/deadly as heart disease by year 2020 [3,4]. Therefore, the need to investigate additional biochemicals, both synthetically and naturally derived, for antidepressant properties is critically needed.

Ethanol withdrawal results in some behavioral abnormalities that parallel symptoms observed in human anxiety/depression and some antidepressants can reverse these symptoms. Associated behavioral deficits in laboratory animals can be reversed by antidepressant treatment, suggesting ethanol withdrawal provides a basis for an animal model of depression [2,5]. Among the numerous neurotransmitter systems implicated in the pharmacological effects of ethanol, the serotonergic system has received particular attention. The serotonergic system has been shown to play an important role in the regulation of ethanol intake, preference, and dependence via central mechanisms [6-13]. Selective serotonin re-uptake inhibitors (SSRIs) and some post-synaptic receptor agonists, who increase serotonergic activity in the synaptic space, are effectively used to reverse the symptoms of depression [14,15]. In addition, there is some pharmacologic evidence regarding the efficacy of antidepressants with dopaminergic effects in the treatment of depression [16]. It is generally accepted that dopamine likely contributes significantly to the pathophysiology of depression.

However, the role of dopamine should be understood in the context of existing theories involving other neurotransmitters which may act independently, and interact with dopamine and other neurochemicals, to contribute to depression. It is suggested that the interaction between dopamine and antidepressants occurs via a primary action of antidepressants in hippocampus, amygdala, or the prefrontal cortex, leading to changes in the output to the nucleus accumbens that in turn modulates dopamine receptor sensitivity [17].

Quercetin is a bioflavonoid (tannin subclass) and has a phenylpropanoid-acetate skeleton [18] (Fig. 1). Flavonoids have well-known antioxidant properties, cardiovascular health benefits [19], and antiviral and antibacterial effects. Quercetin is naturally found in a wide variety of plants, many of which humans consume as fruits and vegetables such as: apples, onions, citrus fruits, parsley, sage, tomatoes [20], olive oil, grapes, blueberries, and blackberries [21]. Additionally, several popular plant-derived beverages, such as tea and red wine, are sources of high quercetin concentration [21]. A number of medicinal plant species, including St. John's wort (*Hypericum perforatum*), known for antidepressant properties, are also sources of quercetin [22-24]. Quercetin specifically has the following documented beneficial uses: 1) functions as an anti-inflammatory agent by preventing release of histamine [25]; 2) lowers cholesterol and blood pressure [26]; 3) helps prevent cancer [27]; 4) enhances endurance [28]; 5) improves memory [29], among other properties. Authors have shown that quercetin acts as an antidepressant by protecting dopaminergic and serotonergic neurons [30,31]. Another study on diabetic mice demonstrated that quercetin administered at 50 and 100 mg/kg had similar antidepressant activity to fluoxetine (5mg/kg) and imipramine (15 mg/kg) [30]. Antidepressant effects of quercetin are only recently being illuminated.

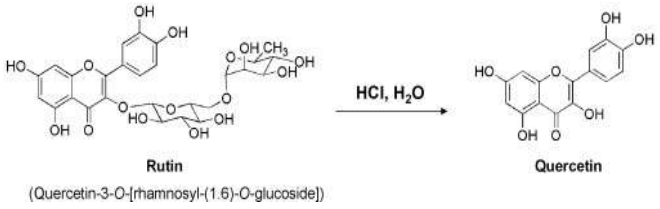

Figure 1. Molecular structure of rutin and quercetin, and the respective conversion process.

Rutin, like quercetin, is a bioflavonoid, and it is comprised of quercetin and the disaccharide rutinose (rhamnose and glucose) [32] (Fig. 1). Certain medicinal plants, such as St. John's wort (*Hypericum perforatum*) contain rutin [23,33,34]; these authors have studied the antidepressant effects of rutin. As a flavonoid, rutin has antioxidant, anticancer, immune system boosting, and cardiovascular protective properties [35,36]. Rutin is reported to work in combination with other flavonoids against depression. Machado et al. [37] indicate that rutin makes serotonin available in the synaptic cleft. Kim et al. [38] state that rutin serves as a source of quercetin to promote digestive health, and Schulz et al. [39] describe the metabolic breakdown of quercetin in the form of glycosides like rutin through enzymatic hydrolysis in the small intestine of humans. Khan et al. [40] report, using male Wistar rats as model organisms, that rutin counteracts behaviors associated with Parkinson's disease by protecting dopaminergic neurons.

The goals of our study were: 1) to quantitatively measure changes in zebrafish behavior following exposure to quercetin and rutin, and 2) to quantitatively measure changes in neuronal morphology and distribution in zebrafish brain nuclei following exposure to quercetin and rutin.

2. Methods

2.1. Animals and Housing

Adult zebrafish (>90 days; 50/50 male/female) were acquired from a local commercial distributer (Pets Paradise 4 U, Enterprise, AL, USA). The fish were housed in 1L tanks (Thoren Aquatic Systems, Inc. Hazleton, Pennsylvania, USA; recirculating high-density rack system) in groups of 5 fish per tank. The tanks were filled with de-ionized water treated with Prime Freshwater® concentrated conditioner. The tank water was filtered using mechanical (sponge), chemical (activated carbon), and biological filtration units. The water temperature was maintained at 25-27°C. Fish were kept on a 10/14 light-dark cycle (lights on 9AM, off at 7PM). Fish were fed a mixture of ground flake food (Tetramin Tropical Flakes; Tetra USA, Blacksburg, VA). All fish were maintained and procedures were performed in accordance with the Institutional Animal Care and Use Committee of Troy University, Troy, AL, USA.

2.2. Treatments

All of the fish were naive and given at least 7 days to adapt to the laboratory environment. Fish were randomly sorted into treatment groups (n = 10) and controls (n = 10) so that there was no confounding of breeding or holding conditions with respect to pharmacological treatment. Instead of continuous exposure, we adopted a regimen of intermittent treatment exposure, as this allows better control of the dose. A pilot dose-escalation study was conducted to ensure the optimal dose for all of the below treatments.

Behavioral measurements were tested following chronic intermittent exposure to dissolved 3% ethanol (Fisher Scientific, USA) in a 1L beaker filled with 900 mL tank water. Fish were gently transferred by net from their home tanks into the beaker for 1hour. This dosing regimen

results in stable blood ethanol concentrations, based on prior pilot studies. After 2 weeks of daily exposure, the fish underwent abstinence/withdrawal from ethanol in their home tanks. During this period, they were examined in the Novel Tank Diving Test on the 2nd and 6th day of withdrawal. No physical abnormalities were observed during the test period. Based on previous studies [5], it was found that the 6th and 7th day of withdrawal resulted in the most robust behavioral effects, i.e., maximum levels of anxiety/depression. Therefore, for purposes of this paper, only these time points are shown.

Three and 10% of both quercetin and rutin, dissolved in 0.5% DMSO, (Sigma-Aldrich, USA) were administered daily for 2 weeks immediately following ethanol withdrawal, to assess possible behavioral and neurochemical alterations. Fluoxetine (100 µg/L) (Sigma–Aldrich, USA) was also administered beginning the day ethanol was discontinued after the 2 week exposure period, to serve as a "gold standard" comparison for quercetin and rutin, in order to determine if the emergence of anxiety/depression-like behavior during abstinence can be muted/prevented. Behavioral testing was performed at 1 and 2 week time points during the fluoxetine, quercetin and rutin exposure period. Following behavioral testing, fish were euthanized in 500 mg/L Tricaine (Sigma-Aldrich, USA).

2.3. Behavioral Analysis

In order to assess anxiety/depressive-like behavior, a standardized behavioral assay was performed. Behavioral testing took place during the light phase, between 9AM and 5PM. The novel tank diving response of zebrafish can provide considerable insight into anxiety/depressive behaviors. The diving response to dwell in the bottom of a novel tank and subsequently explore higher levels of the tank resembles thigmotaxis (position choice along the wall) seen in rodents upon initial presentation into a novel open field that is commonly interpreted as anxiety. To prevent human error and variability in recording of the zebrafish movements during this assay, an iPhone® App video tracking system developed in our lab was employed to record the movements [5]. These tracking system tools are able to provide several endpoints that human observation cannot; tota distance traveled, velocity, and distance traveled in the top/bottom portion, and a traceable map of the path that the fish take.

The fish were placed individually in a 5 L tank (approximately 20 cm H x 30 cm TW) maximally filled with aquarium treated tap water. The tank was divided into two equal horizontal halves, marked by a Sharpie® on the outside of the tank. The tank rested on a stable, level surface, and all environmental distractions were kept to a minimum. An iPhone® was placed approximately 30 cm in front of the novel tank to ensure that the novel tank was within the vision range of the iPhone camera. A standard letter-sized yellow (21.59 cm x 27.94 cm) sheet of paper was placed behind and on the sides of the novel tank to ensure a uniform background for the video analysis. A 75-watt light bulb was placed behind the yellow screen to boost contrast of the background and the fish. Each fish was transported from the home tank to the novel tank with care to reduce net-stress. Once placed in the novel tank, zebrafish movement was recorded for 5 minutes. The zebrafish was then removed from the novel tank and the brain extracted. Immunohistochemistry, described below, commenced.

2.4. Immunohistochemistry

Serotonin and dopamine are ubiquitous neurotransmitters; however, they have significant presence in the hippocampus. Almost all serotonin and dopamine receptor subtypes are expressed in hippocampus, which implicates an intricate modulating system. In this study, immunohistochemical staining for dopaminergic and serotoninergic neurons in the telencephalon of the zebrafish, which is homologous to the hippocampus, was used to detect changes in neuronal numbers following pharmacological treatment.

For the immunohistochemistry analysis, brains were isolated and fixed in 4% paraformaldehyde overnight at 4°C. After rinses in phosphate-buffered saline (PBS) (pH 7.4), the brains were incubated in 30% sucrose. Sections were cut on a cryostat at 10 µm. Antibody characterization was via monoclonal antibodies against 5HT (Abgent, San Diego, CA). Differences in immunoreactive 5HT neurons were tested using an established immunohistochemistry procedure in our lab. Briefly, the brain was prepared by making coronal slices, with a razor blade, at the caudal-most region of the olfactory bulbs and the rostral-most portion of the brain. The right side of the brain was then "notched", adjacent to the midline, rostro-caudally, to assist with tissue orientation. Afterwards, the brain was rapidly frozen on a cryostat stage. The brain was subsequently prepared for cutting with application of OTC (Tissue-Tek, Miles Inc., Elkhart, IN) over the outer surface of the brain. Two consecutive 10µm sections each were placed on two separate sets of 0.5% gelatin-coated slides (12 slides per stain for each case), one control/one treated. This process was repeated every 25µm until tissue was acquired for 20 slides. For each case, the matched control brain and treated brain were placed adjacent to one another on each of the slides. This insured that sections from the treated and matched vehicle control brains were always exposed to identical tissue processing conditions. Sections were counter-balanced between cases to prevent possible position effects. In addition, each set of slides from a given case includes an additional slide used as an omission control for non-specific staining. After cutting the tissue, a circle was made around each section using a "PAP" pen, hydrophobic marker (Research Products, Mount Prospect, IL), to facili-

tate the containment of incubation solutions placed on the sections.

Brain tissue was processed by the avidin-biotin-peroxidase complex (ABC) method originally described by Hsu (1991). Slides were gently rinsed with PBS and placed in Coplin jars filled with PBS on a shaker table (0.5 RPM) for 10 minutes. Slides were then dried using Kimwipes and incubated for 20 minutes in a 10% blocking serum (10% normal goat serum (Vector Labs, Burlingame, CA) in PBS with 0.15% Triton-X-100) in a humidity chamber. Slides were then dried again and incubated in well characterized, commercially available primary antibody to 5HT (Abgent, San Diego, CA). Primary antibodies were diluted with a solution of 1% goat serum in PBS with 0.15% Triton-X-100 to 600:1 for 18 hours in a humidity chamber. Omission control sections for the case were processed in identical solutions but without primary antibody exposure. At the end of the primary antibody incubation, slides were gently rinsed and placed in Coplin jars for 3 X 5 minute wash baths (PBS). The tissue was then incubated for 45 minutes in a biotinylated secondary antibody solution, biotinylated goat anti-rabbit secondary antibody solution (Vector Labs, Burlingame, CA) in 1% diluent with 0.15% Trition-X-100 in an incubation chamber. Following the biotinylated antibody incubation, the slides were rinsed (3 X 5 minute wash baths (PBS)), then incubated in 3% hydrogen peroxide in PBS for 10 minutes (to quench endogenous peroxidase activity), then rinsed (3 X 5 minute wash baths (PBS)). Subsequently, the sections were incubated for 45 minutes in an avidin-biotin peroxidase complex solution (ABC elite kit, Vector laboratories). At the end of the ABC incubation, the slides were rinsed again (3 X 5 minute wash baths (PBS)). Immunoreactivity was revealed by incubation in diaminobenzidine (DAB) solution (0.05% diaminobenzidine and 66 µl of 30% hydrogen peroxide in PBS). Sections were incubated in DAB for 5 minutes. Following DAB incubation, slides were rinsed (3 X 5 minute wash baths (PBS)). Sections were then dehydrated through a series of ethanol baths (70%, 80%, 95%, 100%), cleared in xylene, and coverslipped with Permount®.

2.5. Image Analysis

The digital images captured from each section were analyzed using the "Histogram" tool of Adobe Photoshop© software. For any selected area of pixels from a digital photograph, this function can determine the mean gray scale value (0-255, where 0 is black and 255 is white) and the number of pixels that are darker than a specified grayscale value. All images were converted to grayscale and a mean minimum grayscale value was determined for immunolabeled varicosities. This grayscale value was determined from all vehicle control brains. All pixels darker than this minimum value would subsequently be counted as positive immunostaining. The mean minimum grayscale value was determined by finding the mean grayscale value of two immunolabled varicosities per field (loci), judged as the minimum acceptable for measurement. The grand section mean across the four fields of each section was then determined. Then a grand mean for the brain was determined across each of the grand section means. The minimum acceptable grayscale value, calculated from all the vehicle control brains, was then used to make pixel counts on both the vehicle control and treated brains. The number of pixels that had a value less than, i.e. darker than, the minimum acceptable grayscale value was recorded for each field within a brain section. The count for each field was used to calculate a mean hippocampal pixel count of immunopositive labeling for each section. The mean pixel count for each treated section on a single slide was compared with its adjacent, matched vehicle-treated section. The mean of these differences was then calculated across slides for a given case (case =one treated brain and matched vehicle control brain). These mean differences in pixel counts were tested for their difference from zero.

2.6. Data Analysis

All analyses were carried out using SPSS (version 17 for Windows, SPSS Inc, Chicago, IL, USA). A completely randomized statistical design was used with behavior as the response and time as the categorical variable. Non-normal data were transformed to meet the assumptions of the ANOVA procedure. Data that could not be transformed to meet the assumptions were ranked prior to analysis. A Tukey–Kramer post hoc mean comparison test was used to evaluate differences (α=0.05) between time periods in the event of a significant F statistic. Results were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Behavioral Measures

Based on the novel tank diving test results (Fig. 2), zebrafish spent considerable periods of time in the upper half of the tank following administration of both quercetin and rutin. This activity correlates with more anti-depressant/anxiolytic behavior, whereas staying in the lower half of the tank corresponds with less anti-depressant/anxiolytic behavior. In Figs. 2A and 2B, it is clearly shown that antidepressant effects of 3% and 10% quercetin are comparable to treatment with fluoxetine and significantly better than the ethanol withdrawal group. Some behavioral improvement following treatment with 3% and 10% rutin was observed compared to the ethanol withdrawal group (Figs. 2C & 2D); however, less anti-depressant/anxiolytic behavior was exhibited by zebrafish following exposure to rutin compared to fluoxetine. ANOVA revealed a significant treatment (quercetin and rutin) effect ($F(3, 22) = 6.683$, $p > 0.05$).

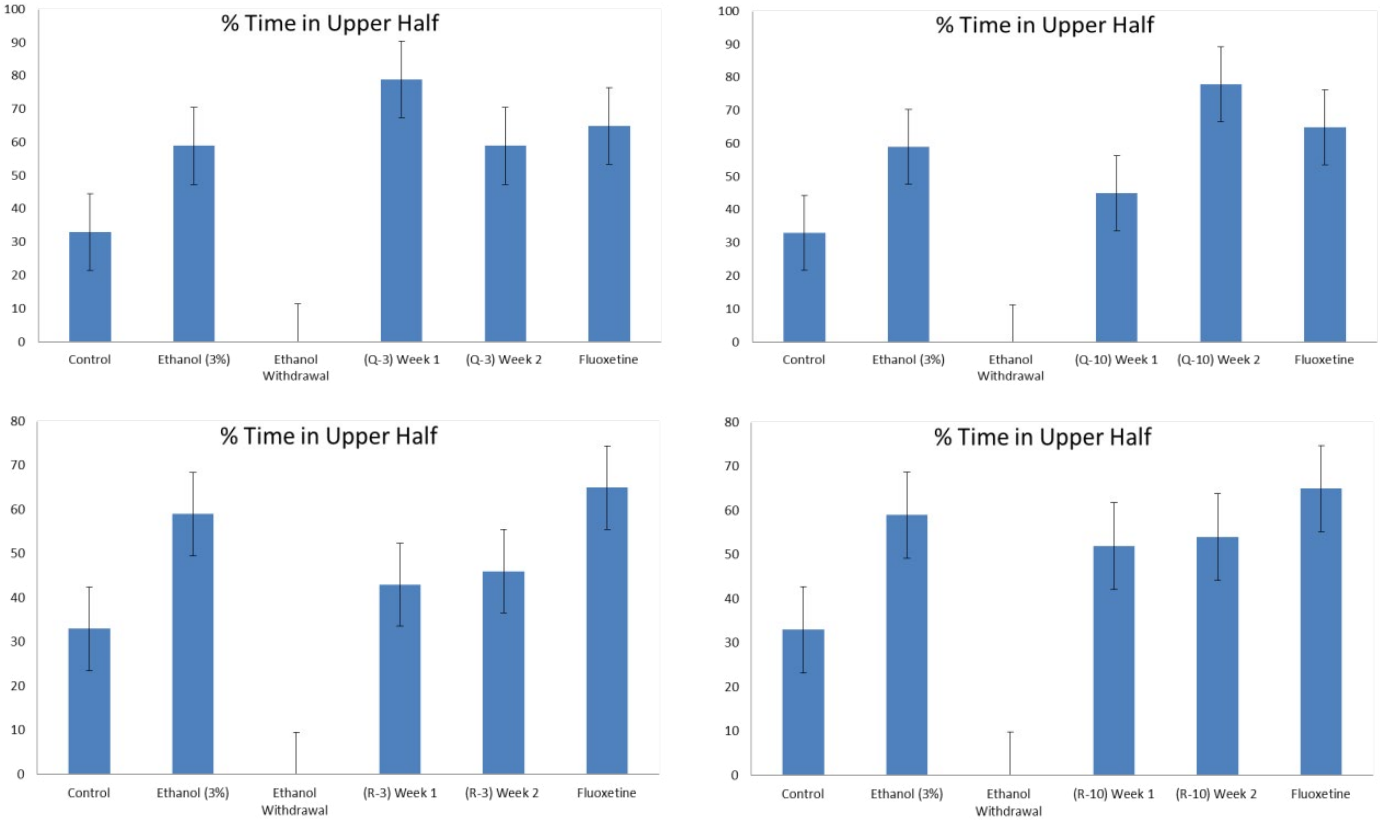

Figure 2. Behavioral effects of chronic intermittent exposure to 3% ethanol daily for 2 weeks, subsequent withdrawal, and fluoxetine exposure in the Novel Tank Diving Test. (A) 3% quercetin (Q-3), (B) 10% quercetin (Q-10), (C) 3% rutin (R-3), (D) 10% rutin (R-10). Data are presented as mean ± SEM; $p < 0.05$ (n = 10 per group).

3.2. Immunohistochemistry

As shown in Figures 3 and 4, significant differences in dopamine immunostaining were seen among quercetin and rutin treatment, as compared to fluoxetine and withdrawal groups. ANOVA revealed a significant treatment (quercetin and rutin) effect ($F(3, 22) = 7.843$, $p > 0.05$). Histogram images resulting from immunohistochemistry (Fig. 3 A-D) show sections through the telencephalon, a rudimentary hippocampal brain region in zebrafish, specifically stained for dopaminergic terminals.

This region of zebrafish brain following ethanol withdrawal treatment (Fig. 3C) appears nearly devoid of immunoreactive dopaminergic terminals. Zebrafish brain subsequent to exposure to fluoxetine, known to affect serotonin levels, also shows little dopaminergic reactivity (Fig. 3D). However, zebrafish brains treated with both 10% quercetin (Fig. 3A) and 10% rutin (Fig.3B) exhibit significant upregulation of dopaminergic immunopositive staining. Similarly for serotonin, following treatment with both quercetin and rutin, a significant treatment effect resulted ($F(3, 22) = 7.219$, $p > 0.05$).

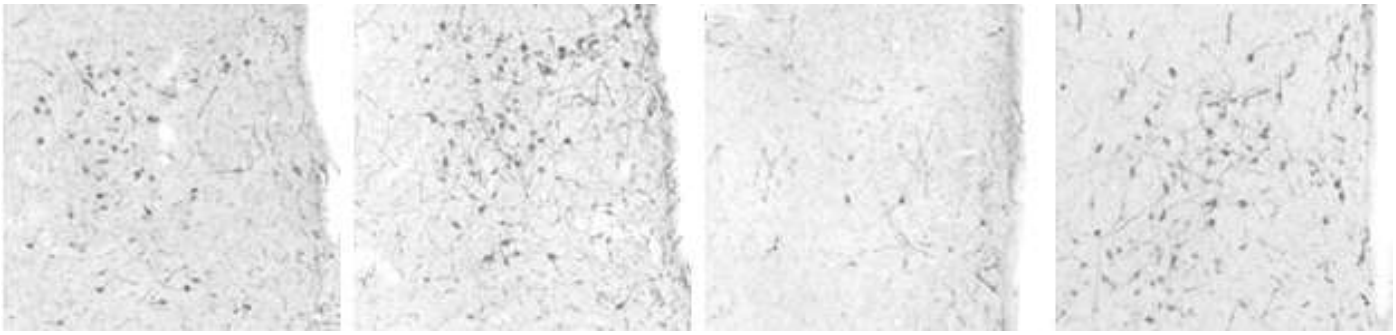

Figure 3. Representative images of coronal sections through the telencephalon (hippocampus) showing an example of immunopositive staining of dopamine terminals. (A) 10% quercetin (week 2), (B) 10% rutin (week 2), (C) ethanol withdrawal, (D) fluoxetine (week 2). Images (100x).

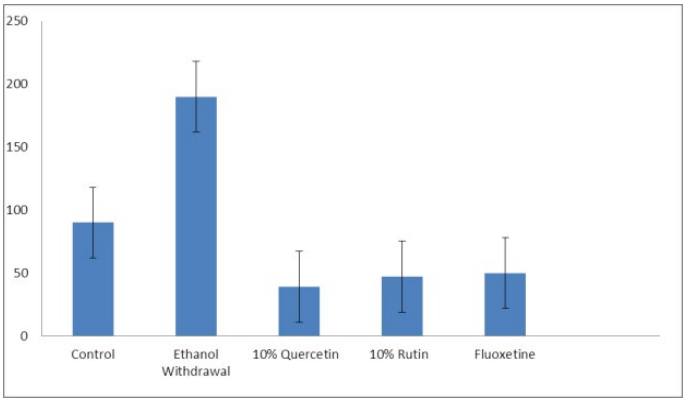

Figure 4. Quantitative image analysis of coronal sections through the telencephalon (hippocampus) of immunopositive dopamine terminals. Mean gray scale values represented on the y axis (0-255, where 0 is black and 255 is white) are a function of immunolabeled varicosities.

4. Discussion

Several authors have reported the neuroprotective effects of quercetin in humans [29,31] and have even more definitively shown how quercetin has positive effects on serotonergic neurons [30] and dopaminergic neurons [31] possibly leading to the potential use of therapeutic doses of quercetin as additional treatments for conditions such as Parkinson's disease and Alzheimer's disease [41]. Certain medicinal plants, including St. John's wort (*Hypericum perforatum*), have bioactive compounds such as hyperforin and quercetin that are known to improve cognitive function and memory and have been shown to disassemble amyloid beta-peptide aggregates associated with Alzheimer's disease [41,42]. Results of this study obtained through using immunohistochemical techniques (Fig. 3A) strongly support the role of quercetin in protecting dopaminergic neurons. The novel tank diving test results clearly show the antidepressant effects of quercetin administered to zebrafish (Figs. 2A & 2B). Based on the results of this study, it is hypothesized that quercetin is functioning as an antidepressant by affecting levels of both dopamine and serotonin, and further demonstrates the connection between an imbalance in dopamine levels and depression.

As previously reported, rutin is known to affect levels of serotonin [37], which would assist in reducing depressive behaviors and would also correlate with promotion of digestive health [38]. The latter authors also point to the metabolic connection between rutin and quercetin. As shown in Fig. 1, rutin can be converted to quercetin through enzymatic hydrolysis in the small intestine [39,43] and the action of HCl in humans. Behavioral results using the novel tank diving test on zebrafish following administration of rutin did show improvement in that anxiolytic/depressive actions were less severe than in control, but improvement was not as pronounced as behavioral results using the novel tank diving test on zebrafish following administration of quercetin, which was comparable to treatment with fluoxetine. It is hypothesized that treatment with rutin was not as effective as treatment with quercetin within the experimental time-frame due to the fact that rutin is a larger molecule that after metabolic breakdown serves as a source of quercetin [39,43] and due to a lack of gastric HCl in zebrafish [44]. However, immunohistochemical techniques producing images of the hippocampal equivalent regions in zebrafish clearly demonstrate the presence of significant levels of both serotonin and dopamine following treatment with rutin (Fig. 3B). The effects of rutin on dopamine are not well documented. Therefore, the zebrafish telencephalon brain regions in Fig. 3B showing obvious dopamine terminals confirm the action of rutin on dopamine. The work of Khan et al. [40] supports these findings; they state that rutin has a protective effect on dopaminergic neurons.

The results of this study show that both rutin and quercetin cause a significant upregulation of dopaminergic and serotonergic neuronal changes in zebrafish, whereas the selective serotonin reuptake inhibitor fluoxetine, as anticipated, did not significantly increase dopamine levels in zebrafish. The significant changes caused by rutin and quercetin suggest that these flavonoids contribute to the antidepressant effects of certain medicinal plant extracts, such as those obtained from St. John's wort (*Hypericum perforatum*). In conclusion, the findings of this study suggest that rutin and quercetin are both effective in the serotonergic and dopaminergic systems relevant for antidepressant activity. The findings reported here provide further insight into the mechanism of antidepressant action of medicinal plants like *H. perforatum* and suggest that rutin and quercetin are constituents mediating some of the documented antidepressant activity of *H. perforatum* and other medicinal plants in which these compounds are found.

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