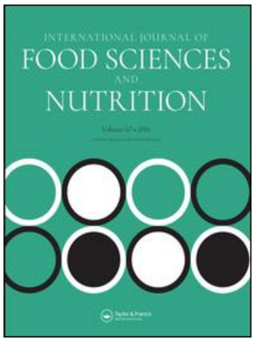

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RESEARCH ARTICLE

Effect of blackberry juice (*Rubus fruticosus L.*) on anxiety-like behaviour in Wistar rats

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ABSTRACT

The present study evaluated the effects of blackberry juice that is rich in different concentrations of anthocyanins and polyphenols (2.6 mg/kg anthocyanins, 14.57 mg/kg polyphenols; 5.83 mg/kg anthocyanins, 27.10 mg/kg polyphenols; 10.57 mg/kg anthocyanins, 38.40 mg/kg polyphenols) on anxiety-like behaviour in Wistar rats. The rats were treated with blackberry juice for 21 days and then tested in the elevated plus maze, locomotor activity test and forced swim test. The results were compared with a reference anxiolytic drug diazepam (2.0 mg/kg) and vehicle (8.7 ml/kg). The intermediate dose of blackberry juice exerted an anxiolytic-like effect that was similar to diazepam, without affecting locomotive activity. The low and high doses of blackberry juice exerted no significant effects on anxiety-like behaviour compared with vehicle. In the forced swim test, both the high and intermediate doses of blackberry juice reduced total immobility time, suggesting a protective effect against behavioural changes that are induced by acute stress. These findings suggest a potential therapeutic effect of blackberry juice on anxiety that is associated with a stressful event.

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Blackberry; anthocyanins; anxiety; elevated plus maze; locomotor activity test; forced swim test

Introduction

Anxiety is one of the most common psychiatric disorders worldwide. It is a chronic condition with negative effects on those who experience it and their immediate social network (Rosas-Santiago et al. 2017). Approximately, 10% of the world's population has been affected by anxiety (World Health Organization 2016). The treatment of anxiety disorders is often based on the use of pharmaceuticals, mainly benzodiazepines and some antidepressant drugs with anxiolytic activity, such as selective serotonin reuptake inhibitors (Baronet-Jordana 2010; Lakhan and Vieira 2010). Unfortunately, these treatments may be accompanied by side effects, and tolerance and drug dependence may develop in the long term (Bateson 2002; Carlini 2003; Ravindran and Stein 2010). Some patients complement pharmacological therapies with alternative treatments that are based on natural compounds. Some of these natural compounds have been evaluated preclinically as potential anxiolytics, such as crude water-based extracts of three species of *Montanoa* (Carro-Juárez et al. 2012; Rodríguez-Landa et al. 2014; Sollozo-Dupont et al. 2015) and other active constituents of a vegetal origin, such as genistein (Rodríguez-Landa et al. 2009), polyphenols (Viola et al. 2000; Zhang et al. 2012) and anthocyanins (Valcheva-Kuzmanova et al. 2016), among others.

Preclinical studies have reported evidence of the biological actions of polyphenols and anthocyanins, highlighting their anticarcinogenic (Kamei et al. 1998; Kraft et al. 2005), cellular antiinflammatory (Hagiwara et al. 2002), hypoglycaemic (Shipp and Abdel-Aal 2010) and ocular antiinflammatory (Ohgami et al. 2005) effects. In the brain, these compounds are able to penetrate the hematoencephalic membrane and act on diverse cerebral structures, such as the hippocampus and cortex (Andres-Lacueva et al. 2005; Talavéra et al. 2005). These actions have been shown to promote neuroprotective effects (Kelsey et al. 2011) and improve memory function in both preclinical (Andres-Lacueva et al. 2005) and clinical (Krikorian et al. 2010) research.

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Anthocyanins and polyphenols from such fruits as blueberries (Ramirez et al. 2005), mulberries (Yadav et al. 2008) and chokeberries (Valcheva-Kuzmanova and Zhelyazkova-Savova 2009; Tomić et al. 2016; Valcheva-Kuzmanova et al. 2016) produce anxiolytic effects in consumers, in addition to their well-studied benefits as antioxidants and neuroprotective agents (Barros et al. 2006; Shukitt-Hale et al. 2008; Dreiseitel et al. 2009). They are also able to inhibit monoamine oxidases and stabilise serotonin, norepinephrine, and dopamine levels, alterations of which are related to the aetiology of anxiety disorders (Dreiseitel et al. 2009).

Blackberries (*Rubus fruticosus*, Rosaceae family) contain high levels of polyphenols and anthocyanins (Kaume et al. 2012). Therefore, they are considered a highly antioxidant food (Cho et al. 2004). To date, however, only a few controlled studies have evaluated the effects of blackberries on emotional states in rats. Riaz et al. (2014) evaluated the effects of a methanolic extract of *Rubus fruticosus* L. in rats and reported an anxiolytic-like effect in the hole board test and an antidepressant-like effect in the forced swim test. The present study evaluated the effects of blackberry juice that was ultrasonically processed to be rich in antioxidants and polyphenols on anxiety-like behaviour, locomotor activity, and the behavioural response to acute stress in male Wistar rats. We sought to identify a potential anxiolytic effect of blackberry juice that may help control anxiety symptoms that are associated with a stressful situation.

Methods

Ethics

All of the experimental procedures were performed according to international ethical guidelines based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals and official Mexican Guidelines for the Use and Care of Laboratory Animals (NOM-062-ZOO-1999). All efforts were made to minimise animal discomfort during the study.

Animals

Forty-five male Wistar rats, weighing 200–250 g at the beginning of the study, were maintained under a 12 h/12 h light/dark cycle (light on at 7:00 AM) at 25 °C ± 1 °C. The rats were pair-housed in Plexiglas cages with *ad libitum* access to water and a standard diet.

Blackberry juice

Fresh blackberries (Driscoll's de México®) were obtained from a local market. The fruits were washed with a 0.1% sodium hypochlorite solution and ground for 30 s. Afterward, they were ultrasonically processed according to the method by Esteves-Mar (2016). Ground fruit was placed in a beaker over ice and processed at 40% amplitude for 10 min, with 5 s in on-mode and 5 s in off-mode, using an ultrasonic homogeniser with a 20 kHz frequency and 750 W power (Cole-Parmer Instrumental Company, VCX-750, Vernon Hills, IL). The pulp was then centrifuged at 4000 rpm for 10 min at 10 °C (Hettich Universal 32R). A portion of the resulting juice was lyophilised (FreeZone 4.5 Liter Cascade Benchtop Freeze Dry System) and then mixed with the rest of the juice to increase the concentration of the compounds under study.

Analysis of total polyphenols and monomeric anthocyanins

The Folin and Ciocalteau method for the analysis of polyphenols was used, as described by Singleton and Rossi (1965). A calibration curve was made using gallic acid (12 mg EAG/100 ml) as a reference and read at 730 nm. The results are expressed as mg equivalent of gallic acid/L of sample.

Anthocyanins were quantified by applying the differential pH method as described by Giusti and Wrolstad (2001). The amount of anthocyanins is expressed as mg of cyanidin 3-glycoside/L of sample. All of the samples were analysed in triplicate (Table 1).

Experimental groups

The rats were distributed into five groups ($n = 9$/group): the vehicle group received water (8.7 ml/kg), three groups received different doses of blackberry juice (low: 2.6 mg/kg anthocyanins, 14.57 mg/kg polyphenols; intermediate: 5.83 mg/kg anthocyanins, 27.10 mg/kg polyphenols; high; 10.57 mg/kg anthocyanins, 38.40 mg/kg polyphenols), and one group received 2 mg/kg diazepam (Laboratorios Pisa S.A de C.V., D.F., México) as the reference anxiolytic drug (Contreras et al. 2011; Carro-Juárez et al. 2012).

The blackberry juice doses that were used in the present study were calculated according to Clifford (2000) by considering the recommended daily intake of anthocyanins and the findings of Valcheva-Kuzmanova et al. (2009, 2016), who reported an anxiolytic-like effect of chokeberries (*Aronia melanocarpa*), another fruit from the *Rosaceae* family. From

Table 1. Chemical analysis of blackberry juice in different concentration.

	Total polyphenols (mg GAE/L)	Monomeric anthocyanins (mg C3G/L)
BL	1675 ± 3.51	299 ± 2.31
BM	3116 ± 15.60	671 ± 79.43
BH	4414 ± 31.53	1216 ± 118.20

Values are mean ± DE.
GAE: gallic acid equivalent; C3G7L: cyanidin-3-glucoside; BL: blackberry low dose; BM: blackberry medium dose; BH: blackberry high.

these data, a dose–response curve for blackberry juice was generated. All of the treatments were administered orogastrically for 21 days, with one 8.7 ml/kg dose per day. Diazepam was administered intraperitoneally every day (1 ml/kg volume injected). At the end of the experiment, the rats were consecutively evaluated in the elevated plus maze (5 min), locomotor activity test (5 min) and forced swim test (4 min). Approximately, 2 min elapsed between each behavioural test.

Behavioural tests

Ethics

The tests battery that was used in the present study allowed a reduction of the number of animals used, as recommended by Russell in the 3Rs (refine, reduce, replace).

Elevated plus maze

This test was designed to evaluate and identify substances with potential anxiolytic-like effects (Pellow et al. 1985; Walf and Frye 2007). The elevated plus maze consisted of four arms that were arranged in the shape of a cross. Two of the arms were open (50 cm length and 10 cm width; they had no walls) and were painted white. The other two arms had the same dimensions plus two walls (40 cm height), both painted black. The maze was elevated 50 cm above the floor (Engin and Treit 2007). The test was performed in an isolated room that was illuminated at 40 lux.

The animals were placed in the centre of the apparatus with their head facing an open arm. The test lasted 5 min. The following variables were evaluated: (a) time spent on the open arms (in seconds), (b) number of entries into the open arms, (c) total number of entries into the open and closed arms, (d) percentage of entries into the open arms, (e) anxiety index (AI = 1 – [(time spent on the open arms/test duration)+(entries into the open arms/total number of entries)/2]; Cohen et al. 2013), (f) head-dipping risk behaviour (i.e. when the rat poked out its head toward the floor while standing on an open arm; this response may occur when the body of the animal is located in either the open or closed arms; Griebel et al. 1996; Walf and Frye 2007), and (g) attempts to explore the open arms, immediately followed by an avoidance response (i.e. the animal tries to enter an open arm but then returns to a closed arm). The frequency and time of head-dipping and attempts were quantified.

An entry into any of the arms was only considered when three quarters of the body of the rat were inside the arm. The cage was cleaned with a 30% ethanol solution to remove the scent of previously evaluated rats and avoid any influence on the behaviour of subsequent rats (Gutiérrez-García and Contreras 2002).

Locomotor activity test

The locomotor activity test was performed to evaluate the effects of the treatments on spontaneous locomotor activity and eliminate possible hypo- or hyperactivity that was caused by the treatments, which may influence the rats' behaviour in the elevated plus maze and forced swim test. The test cage was 44 cm × 33 cm × 20 cm. It was constructed of opaque glass and had 12 squares (11 cm × 11 cm) that were drawn on the floor (Rodríguez-Landa et al. 2009). The following variables were evaluated: (a) number of squares crossed (crossings), corresponding to the number of times the rat crossed from one square to another with at least three quarters of its body (this was considered a motor index), (b) time spent rearing (the sum of all periods in which the rat had a vertical posture on its hind legs) and (c) time spent grooming. Grooming included behaviours of self-directed cleaning, such as licking the anogenital area and forelimbs, performing elliptical movements of the forelimbs on the ears, head, body and tail (from head to tail), and any elliptical movements of a small amplitude that were limited to the vibrissae in unilateral or bilateral fashion (Kalueff and Tuohimaa 2004, 2005).

The 5-min locomotor activity test was performed after the elevated plus maze test. At the end of each test, the box was cleaned with a 30% ethanol solution.

Forced swim test

The test consisted of placing the rats individually into a rectangular glass tank (40 cm × 30 cm × 50 cm) that was filled with water at 25 °C ± 1 °C to a depth of 25 cm. The depth of the water did not allow the animal to touch the bottom of the tank with its hind limbs. This test has been used to detect antidepressant- and antistress-like effects of various substances and drugs, such as clomipramine, desipramine, fluoxetine and some natural extracts (Reddy and Kulkarni 1996; Contreras et al. 1998; Contreras et al. 2001; Kalueff and Tuohimaa 2005; Lozano-Hernández et al. 2010; Rodríguez-Landa et al. 2018).

In the present study, the forced swim stress model of Borsini (1995) was used, which has a total duration of 6 min but only the last 4 min are analysed. The only variable that was evaluated was immobility time (i.e. the sum of all periods in which the rat performed only minimal movements that allowed it to float without displacing water). All of the behavioural tests were recorded using a Sony DCR-SR85 camera with a 2000× digital zoom. The variables of interest were evaluated by two independent observers using a programme that was designed *ex profeso* for this analysis. The observers reached 95% agreement in their observations.

Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA) for independent groups, with treatment as the factor, followed by the Tukey *post hoc* test for multiple mean comparisons. The results are expressed as mean ± standard error of the mean, and the level of significance was set at $p < .05$. The assumptions of normality and homogeneity were verified. The data were analysed using MINITAB 17 software. Additionally, to evaluate the discriminative power of the treatments that produced anxiolytic-like effects, a decision tree was constructed. The decision tree is a machine-learning technique that creates classification rules from data. Classification rules are represented as branches of the tree using only those variables that are the most informative to discriminate the variable of interest using entropy as information criteria. WEKA 2.2 data mining software was used to develop the former analysis.

Results

Chemical analysis of blackberry juice

Table 1 shows the concentrations of total polyphenols and monomeric anthocyanins that were contained in the processed blackberry juice. The polyphenol content was 86% and 163% higher in the intermediate and high doses compared with the low dose. Anthocyanins presented high stability in the ultrasound and lyophilisation processes, in which the intermediate and high doses were 124% and 307% higher than the low dose.

Elevated plus maze

Time spent on the open arms, number of entries into the open arms, and percentage of entries into the open arms

The number of attempts was significantly different among groups ($F_{4,40} = 4.86$, $p < .003$). The vehicle and high-dose blackberry juice groups exhibited the highest number of attempts (5.78 ± 1.27 and 6.33 ± 0.47, respectively) compared with the other groups. The time spent in attempts was not significantly different among groups ($F_{4,40} = 1.57$, $p = .201$).

The time spent on the open arms was significantly different among groups ($F_{4,40} = 7.47$, $p < .0001$). This variable significantly increased ($p < .05$) in the diazepam and intermediate-dose blackberry juice groups compared with the vehicle and high-dose blackberry juice groups (Figure 1(a)).

The average number of entries into the open arms was not significantly different among groups ($F_{4,40} = 1.3$, $p = .286$): vehicle (4.38 ± 1.33), low-dose blackberry juice (5.44 ± 1.33), intermediate-dose blackberry juice (7.27 ± 1.33), high-dose blackberry juice (6.33 ± 1.33) and diazepam (8.27 ± 1.33). The total number of arm entries (open and closed) was not significantly different among groups ($F_{4,40} = 1.2$, $p = .327$): vehicle (9.16 ± 2.69), low-dose blackberry juice (11.11 ± 2.69), intermediate-dose blackberry juice (14.33 ± 2.69), high-dose blackberry juice (13.11 ± 2.69) and diazepam (16.83 ± 2.69). As expected, no differences were observed in the percentage of entries into the open arms ($F_{4,40} = 0.7$, $p = .596$): vehicle (43.08% ± 4.49%), low-dose blackberry juice (44.34%±4.49%), intermediate-dose blackberry juice (52.46%±4.49%), high-dose blackberry juice (47.01%±4.49%), and diazepam (44.30%±4.49%).

Anxiety index

Significant differences in the AI were observed among groups ($F_{4,40} = 4.14$, $p < .007$). The diazepam and intermediate-dose blackberry juice (5.83 mg/kg anthocyanins, 27.10 mg/kg polyphenols) groups exhibited a significant decrease in the AI compared with the vehicle and high-dose blackberry juice groups (Figure 1(b)).

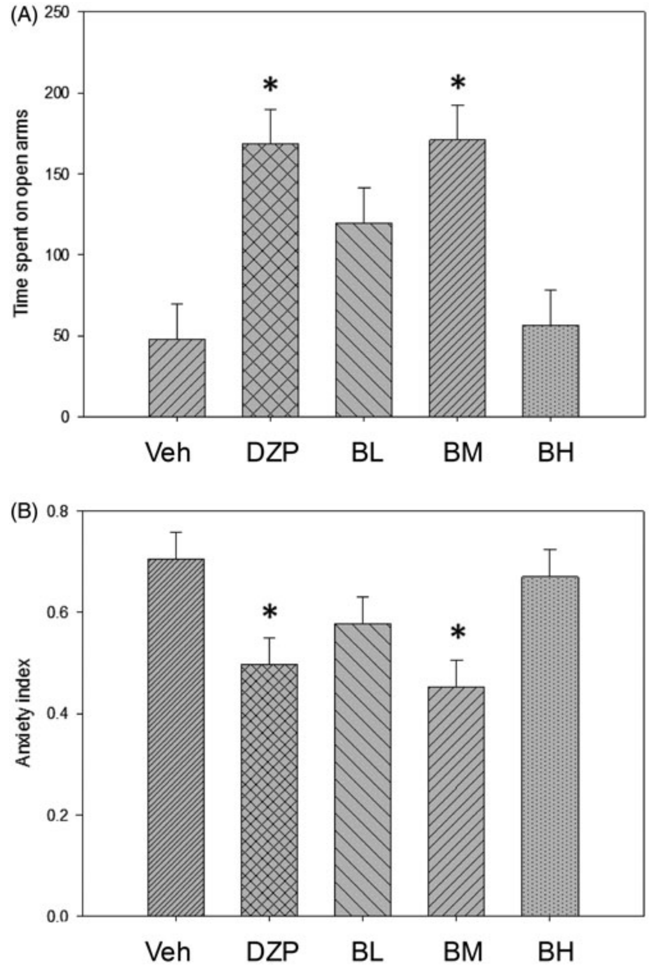

Figure 1. Effects of treatments on the elevated plus maze. (a) Time spent in open arms. (b) Anxiety index. Values are mean ± SE. The number of animals per group was $n = 9$. IT: immobility time; Veh: vehicle; DZP: diazepam; BL: blackberry low; BM: blackberry medium; BH: blackberry high.

Head-dipping and attempts: frequency and time

The time spent in head-dipping was significantly different among groups ($F_{4,40} = 3.53$, $p < .015$). The diazepam group exhibited a significant increase in the number of head-dippings (42.74 ± 9.18) compared with the vehicle group (16.02 ± 5.89) and high-dose blackberry juice group (16.62 ± 5.79). No significant differences were observed in the number of head-dippings ($F_{4,40} = 3.12$).

The frequency of attempts was significantly different among groups ($F_{4,40} = 4.86$, $p < .003$). The vehicle and high-dose blackberry juice groups had the highest frequency of attempts (5.78 ± 1.27 and 6.33 ± 0.47, respectively) compared with the other groups. No significant differences in the time spent in attempts were found among groups ($F_{4,40} = 1.57$, $p = .201$).

Decision tree

To analyse the relationship among the vehicle, diazepam and blackberry juice groups, a decision tree was constructed using the time spent on the open arms and time spent in head-dippings as variables to predict (classify) the groups in which each rat belongs. As shown in Figure 2, all cases of the diazepam and intermediate-dose blackberry juice groups were grouped in the right branch of the tree, suggesting that both groups had similar values.

The confusions matrix shows the number of occurrences in which the decision tree is able to classify the true classes. The confusion matrix revealed that the diazepam and intermediate-dose blackberry juice groups were mainly classified as the same type based on their similarities (Table 2).

Locomotor activity test

The effects of the treatments are shown in Table 3.

Crossings

No significant differences in the number of crossings were observed among groups ($F_{4,40} = 0.11$, $p = .979$).

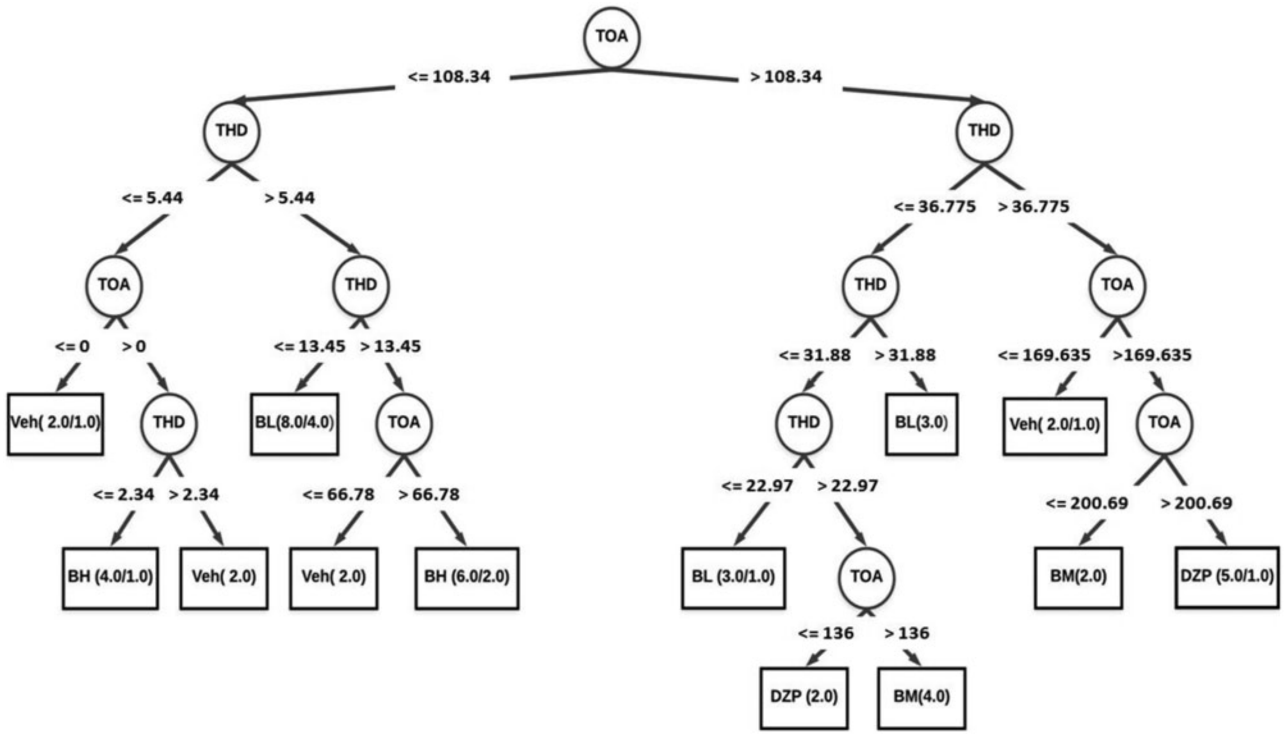

Figure 2. Decision tree constructed from the *time spent in open arms* and *time spent in head-dipping* variables. TOA: time in open arms; THD: time spend in *head-dipping*.

Table 2. Confusion matrix generated by the decision tree.

	Classified				
True class	Veh	DZP	BL	BM	BH
Veh	1	2	1	0	5
DZP	0	**3**	1	**4**	1
BL	2	0	2	3	2
BM	1	**3**	1	**3**	1
BH	3	2	2	0	2

Veh: vehicle; DZP: diazepam; BL: blackberry low; BM: blackberry medium; BH: blackberry high.
The confusions matrix shows the number of occurrences that the decision tree is able to classify the true classes.

Table 3. Variables analysed in open field test.

Variable	Veh	DZP	BL *n*= 9	BM	BH	F	*p* Value
C	45.39 ± 5.18	49.17 ± 7.73	45.22 ± 8.66	47.89 ± 5.12	50.00 ± 5.72	0.11	.98
GF	2.00 ± 0.44	1.78 ± 0.40	1.67 ± 0.41	2.56 ± 0.53	1.89 ± 0.48	0.58	.68
GD	38.06 ± 9.84	17.20 ± 4.26	17.31 ± 4.13	21.68 ± 3.67	21.78 ± 5.02	2.17	.09
RF	25.22 ± 2.55	30.56 ± 3.83	26.11 ± 4.40	29.67 ± 2.07	22.78 ± 2.97	0.97	.44
RD	84.09 ± 8.62	89.02 ± 14.18	67.33 ± 12.71	95.66 ± 7.85	75.67 ± 9.51	1.05	.39

Values are mean ± SE.
C: crossing; GF: grooming frequency; GD: grooming duration; RF: rearing frequency; RD: rearing duration; Veh: vehicle; DZP: diazepam; BL: blackberry low; BM: blackberry medium, BH: blackberry high.

Grooming: frequency and time

No significant differences were found among groups in the frequency of grooming ($F_{4,40} = 0.58$, $p = .681$) or time spent grooming ($F_{4,40} = 2.17$, $p = .09$).

Rearing: frequency and time

No significant differences were found among groups in the frequency of rearing ($F_{4,40} = 0.97$, $p = .437$) or time spent rearing ($F_{4,40} = 1.05$, $p = .394$).

Forced swim test

Significant differences in immobility time were observed among groups ($F_{4,40} = 3.96$, $p < .008$). As expected, the *post hoc* test showed that the intermediate-dose (5.83 mg/kg anthocyanins, 27.10 mg/kg polyphenols) and high-dose (10.57 mg/kg anthocyanins, 38.40 mg/kg polyphenols) blackberry juice groups exhibited a significant decrease in immobility time compared with the vehicle and diazepam groups (Figure 3).

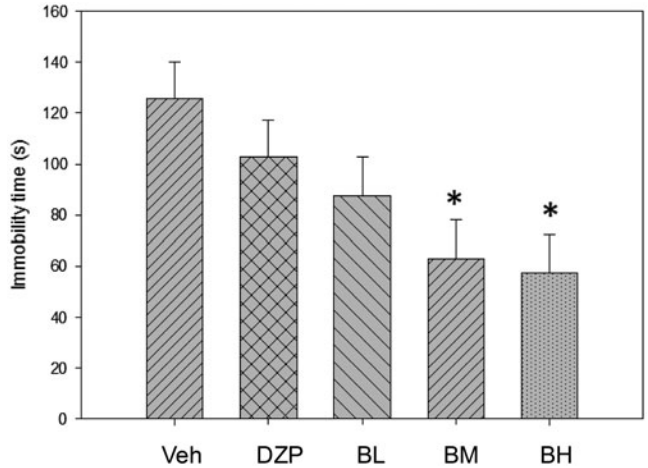

Figure 3. Forced swimming test of vehicle, diazepam and blackberry groups. Values are mean ± SE. The number of animals per group was $n = 9$. IT: immobility time; Veh: vehicle; DZP: diazepam; BL: blackberry low; BM: blackberry medium; BH: blackberry high.

Discussion

In the present study, the effects of different doses of blackberry juice on anxiety-like behaviour were evaluated in the elevated plus maze, locomotor activity test and forced swim test. The intermediate dose of blackberry juice (5.83 mg/kg anthocyanins, 27.10 mg/kg polyphenols) produced an anxiolytic-like effect that was similar to diazepam in the elevated plus maze. The intermediate dose and high dose (10.57 mg/kg anthocyanins, 38.40 mg/kg polyphenols) of blackberry juice exerted antistress-like effects in the forced swim test.

The elevated plus maze is designed to measure anxiety levels and is one of the most commonly used tests to explore potential anxiolytic effects of drugs (Fernández-Guasti et al. 2001; Walf and Frye 2007). This test subjects rodents to aversive stimuli (i.e. causes acrophobia and agoraphobia). Rodents tend to remain on the open arms for less time compared with the closed arms (Singh et al. 2017). In the present study, only the diazepam and intermediate-dose blackberry juice groups exhibited significant increases in the time spent on the open arms and number of entries into the open arms, suggesting lower levels of anxiety (Pellow and File 1986; Engin and Treit 2007). This reduction has also been reported for selective serotonin reuptake inhibitors (Kaminska and Rogoz 2016), neurosteroids (Zhang et al. 2017), fatty acids (Bernal-Morales et al. 2017) and some flavonoids (Bouayed 2010).

The formula that was proposed by Cohen et al. (2013) was used to calculate the AI, which provides a general tendency of the grade of anxiety from 0 to 1, where values near 1 indicate higher levels of anxiety. In the present study, the intermediate-dose blackberry juice group exhibited a reduction of the AI that was similar to the diazepam group. This finding is similar to Contreras et al. (2014) with anxiolytic substances, indicating an anxiolytic-like effect.

Head-dipping behaviour reflects the evaluation of risk and decreases in animals under conditions of danger and stress (Campos et al. 2013). In the present study, head-dipping behaviour increased in the intermediate-dose blackberry juice group, but this increase was not statistically significant. In contrast, diazepam significantly increased head-dipping behaviour. In a similar study, Tomić et al. (2016) reported that the *ad libitum* consumption of *Aronia melanocarpa* (a fruit from the Rosaceae family) increased head-dipping behaviour, but this result may not indicate an increase in risk evaluation because the rats presented signs of hyperactivity in the locomotor activity test, which may reflect a false-positive result in this behavioural test.

In the present study, significant differences in attempts were observed among groups, mainly between the vehicle and high-dose blackberry juice group, both of which exhibited an increase in this behaviour. The attempts variable reflects an evaluation of risk in certain situations, and such attempts are exhibited by anxious animals (Rodgers et al. 1997; Carobrez and Bertoglio 2005). This result is consistent with the AI.

In the present study, we did not observe significant effects of the different treatments on locomotor activity, indicating that the effects that were detected in the elevated plus maze were attributable to emotional causes and not to non-specific motor activity (Ramos et al. 2008). This was an important observation. In studies of the effects of cranberry supplements, which are rich in polyphenols and anthocyanins, an anxiolytic-like effect was observed in rats, with no significant changes in locomotion (Ramirez et al. 2005; Barros et al. 2006).

Grooming behaviour is an indicator of the animal's motivation (Kalueff and Tuohimaa 2004), which may increase in mildly stressful situations but is completely eliminated under conditions of severe stress (Perrot-Sinal et al. 2004). Reductions of grooming time are prevented by anxiolytic and antidepressant drugs, which re-establish such behaviour to values of undisturbed animals (Bateson 2002). In the present study, no significant differences in grooming were observed among treatments.

Rearing behaviour in the locomotor activity test is increased by anxiolytic drugs (Kalueff and Tuohimaa 2004). In the present study, only a nonsignificant tendency toward an increase in this variable was observed in the intermediate-dose blackberry juice group. These findings suggest that the effects of blackberry juice that were observed in the elevated plus maze were not caused by changes in spontaneous locomotion but rather by anxiolytic-like effects.

After analysing relationships between groups, we constructed a decision tree. We found that values of the time spent on the open arms that were ≤ 108.34 s only belonged to the vehicle, low-dose blackberry juice, and high-dose blackberry juice groups. In contrast, the intermediate-dose blackberry juice and diazepam groups were grouped together in the end branches on the right side of the decision tree, meaning that both groups had similar features. The first column of the table shows the real values of the class variable to be predicted (i.e. group). The rows of the table show the values that were predicted by the mathematical model (i.e. decision tree). If all of the cases are predicted correctly, then only the main diagonal of the matrix is filled; otherwise, the cells of the matrix can be thought as misclassification errors. That is, the model predicts a value for the class variable that is different form the real value. The confusion matrix reveals that the diazepam and intermediate-dose blackberry juice groups were mainly classified as the same type because of their similarities.

Importantly, the number of rats per group was sufficient to support the anxiolytic-like effect of blackberry juice in the present study. Therefore, increasing the number of animals per group was unnecessary because the principal variable to detect an anxiolytic-like effect in the elevated plus maze (e.g. time spent on the open arms) significantly increased compared with the vehicle group, an effect that was similar to diazepam. This anxiolytic-like effect of blackberry juice was confirmed by the AI, which includes all of the evaluated variables in the elevated plus maze. Therefore, the relatively low number of animals that were included in the present study is consistent with the 3Rs of ethical recommendations for preclinical research (Russell et al. 1959), which has also been reported in previous studies that used the elevated plus maze, even with a lower number of rats (Carro-Juárez et al. 2012; Rodríguez-Landa et al. 2017; Vázquez-León et al. 2018) than in the present study.

A modified forced swim test was performed to complement the other behavioural tests. Borsini (1995) argued that it is possible to evaluate the response to acute stress without generating behavioural despair. As opposed to the method that was proposed by Porsolt, there is no pre-test, and the test lasts only 6 min, a time point at which the development of behavioural despair is questionable. Such a modification of the forced swim test allows researchers to evaluate coping strategies (i.e. increases or decreases in mobility) that are exhibited by rats when they are faced with acute stress. In the present study, the intermediate and high doses of blackberry juice significantly reduced immobility time compared with vehicle, which can be interpreted as a protective effect against behavioural changes that are induced by acute stress (Figure 3). Notably, the high dose of blackberry juice significantly increased immobility time, but such an anxiogenic-like effect was not observed in the elevated plus maze, possibly because of the affinity of some compounds in blackberry juice to other receptors that have been implicated in the stress response, such as serotonin, norepinephrine and dopamine. Some polyphenols have been reported to have actions on these receptor systems (Rodríguez-Landa and Contreras 2000; German-Ponciano et al. 2018).

Based on the present results, blackberry juice appears to have two different effects – anxiolytic and antistress – that are dose-dependent and similar to diazepam (2 mg/kg). The present study did not investigate the possible mechanism of action of blackberry juice. Based on the literature, however, the anxiolytic-like effects of some flavonoids and anthocyanins have been attributed to their affinity for γ-aminobutyric acid-A ($GABA_A$) receptors (Zanoli et al. 2000; Gutierres et al. 2014). Such a mechanism of action could be involved in the effects of blackberry juice that were observed in the present study. The suppression of the anxiolytic-like effect at the highest dose tested may be associated with the overstimulation of $GABA_A$ receptors. A similar phenomenon has been previously reported for other substances, such as benzodiazepines (Bateson 2002; Rosas-Gutiérrez et al.

2013), neurosteroids and flavonoids (German-Ponciano et al. 2018). Further studies are needed to elucidate the precise mechanism of action of blackberry juice and to purify and identify the compounds that are contained in blackberry juice. The constituents of blackberry juice should then be evaluated separately to evaluate possible synergistic or additive effects.

One limitation of the present study was that we did not explore the mechanism of action of the anxiolytic-like effects of blackberry juice. Polyphenols (e.g. anthocyanins and flavonoids) have been reported to exert their anxiolytic-like effects through the stimulation of $GABA_A$ receptors (Zanoli et al. 2000; García-Gutiérrez et al. 2012). Therefore, considering that we identified a high content of these chemical compounds in blackberry juice, the anxiolytic-like effects could be mediated by GABAergic actions. Previous studies have shown that some polyphenols have high affinity for $GABA_A$ receptors (Marder and Paladini 2002; Wang et al. 2002). When GABAergic agonists activate these receptors, Cl^- conductance increases, thus hyperpolarising the neuron and consequently inhibiting neuronal activity. This neurophysiological effect that occurs through $GABA_A$ receptor chloride ion channels is associated with the anxiolytic effects of several substances, including benzodiazepines, barbiturates, psychoactive drugs, some neurosteroids, and flavonoids (Chebib and Johnston 1999; Enna 2007). Additionally, previous studies have reported that diverse substances with antistress effects (e.g. allopregnanolone, progesterone and some flavonoids) reduce immobility time in the forced swim test through actions on $GABA_A$ receptors. Pretreatment with a $GABA_A$ receptor antagonist blocked the reduction of immobility time (Khisti et al. 2000; Estrada-Camarena et al. 2002; Rodríguez-Landa et al. 2007, 2009; Nin et al. 2012). The reduction of immobility in the present study could be related to interactions between $GABA_A$ receptors and the chemical compounds in blackberry juice. However, we cannot discard the possible participation of other neurochemical pathways in the anxiolytic- and antistress-like effects that were observed herein. Further studies are warranted to explore the mechanism of action of the anxiolytic- and antistress-like effects of blackberry juice to better understand the neuropharmacological processes that are involved.

Importantly, the concentrations of active compounds that were produced by ultrasound and lyophilisation appeared to play an important role in the anxiolytic- and antistress-like effects of blackberry juice. Our findings indicate that blackberry juice may be useful for the management of stress and anxiety. Further investigations of the neural processes that are involved in the effects of blackberry juice are required, and the effects of individual bioactive compounds in blackberry juice on anxiety-related behaviours should be evaluated.

Conclusions

The intermediate dose of blackberry juice (5.83 mg/kg anthocyanins, 27.10 mg/kg polyphenols) exerted an anxiolytic-like effect, improving coping strategies at the behavioural level, and these effects were similar to diazepam. These results were complemented by the forced swim test, suggesting that blackberry juice at the intermediate and high doses improves the response to acute stress. Overall, our findings support the potential therapeutic use of this natural substance to help reduce anxiety that is associated with acute stressful events.

Disclosure statement

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