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Biochemical and protective response in *Drosophila melanogaster* (Harwish strain) exposed to different extracts of *Breonadia salicina*

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ABSTRACT

Oxidative stress is an imbalance between oxidants and antioxidants in favour of the oxidants, disrupting redox signalling and control or molecular damage. *Drosophila melanogaster* is an excellent model not only for genetic studies but also for toxicological, neurophysiological, and circadian rhythm studies. The use of *Drosophila melanogaster* in toxicological studies has increased given the genome of flies has homology to the human genome, thus making it a highly predictive model of toxicity in vertebrates. The research investigates the biochemical and protective responses in fruit flies (*Drosophila melanogaster*) when exposed to different extracts of the plant *Breonadia salicina*, which is known for its therapeutic properties but also contains potentially toxic compounds like alkaloids, terpenoids, tannins, and glycosides. Using solvent partition coefficient methods, the plant parts were extracted with solvents like n-hexane, ethyl acetate, and methanol. Phytochemical screenings, antioxidant activities, and acute toxicity tests were conducted, followed by survival assays and biochemical assays on the flies. The exposure to lethal doses of *B. salicina* extracts resulted in increased levels of biochemical markers such as SOD, MDA, GHS, and CAT in the flies, indicating a response to oxidative stress. The study highlights the toxicological effects of *B. salicina*, which led to increased mortality and induction of cell stress markers. The results caution against the indiscriminate use of plant extracts due to their potential toxic effects, emphasizing the need for safety protocols in the medicinal use of plants. This underscores the importance of understanding both the beneficial and harmful aspects of phytochemicals in plants.

Keywords: *Drosophila melanogaster*, probit analysis, percentage survival, lethal concentration.

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INTRODUCTION

There is no existence of life without plants. Plants are the essential foundation of medicine. Some drugs still used today are derived from traditional medicinal herbs (Yadav et al., 2022). Man has been utilizing plants for the maintenance of his health and well-being since antiquity (Iliyasu et al., 2022). Oxidative stress is associated with several pathologies like cardiovascular, neurodegenerative, cancer, and even ageing. It has been suggested that a diet rich in antioxidants would be beneficial to human health, and a lot of interest is focused on the determination of the antioxidant capacity of natural products (López-alarcón and Denicola, 2012). During the last decades, it has been proposed that oxidative stress, defined as the unbalance between reactive oxygen and nitrogen species (ROS/RNS) production and the antioxidant defence, plays a pivotal role in different pathophysiological conditions (López-alarcón and Denicola, 2012). Oxidative stress, originating from an

increase in ROS/RNS production or from a decrease in the antioxidant network, is characterized by the inability of endogenous antioxidants to counteract the oxidative damage on biological targets (López-alarcón and Denicola, 2012). The idea behind antioxidant supplementation is to restore redox cellular status. Some studies have indicated that antioxidants could also have deleterious effects on human health depending on dosage and bioavailability (López-alarcón and Denicola, 2012). The plant kingdom offers a high range of structural diversity using a variety of biochemicals (Yadav et al., 2022). *Breonadia salicina* is widely used in the treatment of cancer, gastrointestinal disease, fever, headaches, arthritis, diabetes, inflamed wounds, and ulcers. It has also been used to protect against diarrhoea (Iliyasu et al., 2022). *B. salicina* bark is used for stomach complaints and as an astringent and root decoctions are used for the treatment of tachycardia (Gaafar et al., 2014). *B. salicina* is a potentially medicinally and eco-nomically valuable plant that has been used for the treatment of many human diseases because of the presence of numerous secondary metabolites. Plant components are also characterized by their ability to prevent the development of certain diseases. The toxicity and adverse effects of conventional and allopathic medicines have also been valuable factors in the sudden increase in population demands and increase in the number of herbal drug manufacturers, as well as a reduction in the use of chemical drugs (Jamshidi-Kia et al., 2018). Almost 50% of the medicines we use today are derived straight from plants, and 25% of the prescription drugs have their genesis from tropical plants. According to WHO, 80% of the world's population relies chiefly on plant-based traditional medicines, especially for their primary healthcare needs (Haq, 2004). Nowadays, herbal medicines are becoming important throughout the world for the prevention and treatment of various diseases because of their impressive therapeutic effects and fewer side effects as compared to modern medicines (Trivedi et al., 2017). Therefore, the use of herbal preparations in traditional medicine is becoming an alternative and can also serve as a replacement for synthetic drugs of doubtful efficacy and safety (Obiora et al., 2019). The research investigates the biochemical and protective responses in fruit flies (*Drosophila melanogaster*) when exposed to different extracts of the plant *Breonadia salicina.*

MATERIALS AND METHODS

Collection of plant parts

In the present study, *B. salicina* was collected from the Bununu district of Tafawa Balewa LGA, Bauchi state, Nigeria. The leaves and stem bark collected were identified morphologically and authenticated by a botanist at Bayero University Kano. The collected leaves were dried at room temperature for pulverization and solvent extraction.

Extraction of plant parts

The extractions of plant parts were done using the Harborne method (1973) of solvent partition coefficient. The extraction was carried out based on solvent polarity using three different solvents n-hexane, ethyl acetate and methanol.

Determination of percentage yield

The percentage yield of the crude extract was determined for each solvent (Mahmood 2009; Parekh and Chanda, 2007). The percentage yield of the aqueous and ethanolic extracts was calculated as thus:

$$\text{Percentage yield} = \frac{\text{Weight of plant extract before extraction}}{\text{Weight of plant extract after extraction}} \times 100$$

The phytochemical methods

The extracts were subjected to phytochemical screening to determine the presence of alkaloids, carbohydrates, flavonoids, saponins, tannins, glycosides (cardiac, steroidal), terpenes/terpenoids, fatty acids, resins using procedures described by Sofowora (2008); Trease and Evans (2002).

In vitro antioxidant activity assay

The effect of Extracts on DPPH radical was estimated using the method of Katalinic et al. (2006). A solution of 0.135 mM DPPH in methanol was prepared, and 1.0 ml of this was mixed with 1.0 ml of different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25mg/ml) of the different Extracts in methanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature 27º C for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as a reference. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \frac{(\text{Absorbance of blank} - \text{Absorbance of samples})}{\text{Absorbance of blank}} \times 100$$

Drosophila melanogaster fly stock selection

The fly *Drosophilla melanogaster* (Harwish) was obtained from the National Species Stock Center (Switzer Land). The flies were maintained and reared on cornmeal medium at a temperature of 23±1 ^{o}C and 60% relative humidity under 12 h dark/light cycle conditions. All the experiments were carried out with the same *D. melanogaster* (Harwish) as described by Abolaji et al. (2014) and Wuyep et al. (2019).

Acute toxicity testing.

The acute toxicity testing was carried out using the methods described by Abolaji et al. (2014) and Wuyep et al. (2020). The fly food was prepared using the ratio of gram per die to obtain food with concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25mg/ml. The acute toxicity was carried out by placing 15 unsex flies (not more than 24 hours old) per vial in triplicates to have a total number of 45 flies per concentration. The survivals of the flies were recorded for seven days and mortality was taken.

Survival assay

Survival assay was carried out using the methods described by Wuyep et al. (2020). Fly flood was prepared using the ratio of grams per diet using the L.C50 of the different extracts of the leaves and stem extracts. Fifteen (15) unsex flies (not more than 24 hours old) per vial in triplicate to have a total of 45 flies per concentration. Mortality was recorded for seven days after which the flies were homogenized in PBS.

Biochemical assays

The activity of some oxidative stress markers, namely Malondialdehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT), and Reduced glutathione (GSH) was measured.

Malondialdehyde (MDA) assay

Malondialdehyde (MDA) formation was determined according to the Ohkawa method (Chen et al., 2010) with modifications, 200 µL of tissues homogenate was mixed with 1.5 ml 20 % acetic acid ph 3.5, with 1.5 ml 0f 0.8 % TBA (Thiobarbituric acid) and 200 µL 8.1 % SDS (Sodium dodecyl sulphate). The mixture was brought up to a volume of 4ml with water and heated in boiling water for 1 hour. After cooling and centrifugation (4000 RPM) at 4 ºc for 10 minutes, the MDA content in the supernatant was measured at 532 nm with a UV 1650PC UV –VIS spectrophotometer. MDA levels were determined from the calibration curve and expressed in nmol/mg of liver protein.

Superoxide dismutase (SOD)

SOD activity was measured by using NBT (Nitro blue tetrazolium) method (Maag et al., 2015). The 3 ml reaction mixture was prepared by adding 50 Mm potassium phosphate buffer (pH 7.8), 13 Mm methionine, 2 µM riboflavin, 0.1 mM EDTA (Ethylenediaminetetraacetic acid), 75 NBT and 50 µL of enzymes extract.

All the tubes were exposed to 400 W bulbs for 15 minutes, and their absorbance read at 560 nm. The 50 % inhibition of the reaction between riboflavin and NBT in the presence of methionine was considered one unit of SOD activity, and it was expressed in units/mg of protein.

Catalase (CAT)

CAT activity was measured by following the standard protocol (Rzezniczak et al., 2011). The activity was measured based on the quantity of the H_2O_2 substrate remaining, often the action of CAT present in the enzyme's extracts. To measure this, 0.4 ml of enzyme extract was mixed with 2.6 ml of phosphate buffer along with 30 % H_2O_2. The activity was measured by determining the decomposition of H_2O_2 at 240 nm. CAT activity was calculated using the millimolar extinction coefficient of 43.6 and expressed in terms of µm/min/mg of protein.

Reduced glutathione (GSH)

The activity of GSH was measured using the DTNB (5,5-Dithio-bis-(2-nitrobenzoic acid) method (Valéria Soares de Araújo Pinho et al., 2014). To measure the enzyme activity, liver cells were homogenized in an ice-cold 10% TCA (Trichloroacetic acid) and 10mM EDTA solution (1:1).

Then homogenate was centrifuged at 5000 rpm. Further enzyme reaction mixture was prepared by adding 200 µL of supernatant, 0.2 M tris-buffer (pH 8.0) and 50 µL of DTNB. The reaction mixture was incubated for 10 minutes at room temperature to get yellow colored complex. The absorbance was read at 412 nm, and the activity was expressed as µg/mg protein.

Estimation of protein

Protein estimation was quantified as per the standard procedure (lowry, 1951). For this 20 of the homogenates were mixed with Lowry's reagent and folin-ciocalteaus

solution. The optical density was measured at 660 nm against blank after incubation. Then the amount of protein in each group was calculated with the BSA (Bovine serum Albumin) standard graph.

Data collection and statistical analysis

Data obtained from in vitro antioxidant activity were subjected to two-way analysis of variance (ANOVA), and probit regression analysis was used to determine the LC50 for the different extracts using GraphPad Prism software; version 8.2. The results obtained were tested for significant differences at a 5% level.

RESULTS AND DISCUSSION

A wide variety of low molecular weight secondary metabolites with biological properties are stored in plants, making them useful to humans. Evidence from in vitro and in vivo studies indicate that phytochemicals such as flavonoids, proanthocyanidins and anthocyanins (Long et al., 2009; Dong et al., 2012; Lopez et al., 2014) can improve lifespan and delay the ageing process. Besides the beneficial effects of plant preparations traditionally used by the human population, the double-sided properties of plant extracts have been verified. In this context, the investigation and further elucidation of such effects become a valuable contribution to the establishment of safety protocols for the use of medicinal plants.

The results of the extraction showed that the methanol stem and leave extracts had the highest percentage yield of 16.11% and 10.31% followed by the ethyl acetate leave extract with 2.92%, n-Hexane stem and leaves extracts with 2.67% and 2.07% and ethyl acetate stem extract had the lowest percentage yield of 0.875%. Similar results have been reported by Dorcas et al. (2024) who evaluated the effects of seasonal variation in the phytochemical composition of *B. salicina* and found that methanol extracts of leaves, stem bark and roots had the highest yield of extracts throughout the different seasons. The difference can be attributed to the difference in the polarity index of the solvents used (Imam et al., 2016).

The result of the phytochemical screening is presented in Table 1. It was observed that the different extracts contained secondary metabolites such as Alkaloids, Tannins, Flavonoids and Phenols. Saponins were found only in the ethylacetate stem extract, methanol stems and leaf extracts. Carbohydrates were only found in the methanol stem and leaf extracts, whereas Terpenoids were absent in these extracts. The results of this finding coincide with the findings of Mahlo et al. (2013), Martins and Nunez (2015), Nvau et al. (2019) and Tihapi et al. (2015), who indicated that different extracts of B.salicina contained triterpenes and saponins in abundance; the stem contains high levels of tannins, while the wood is rich in polyphenols and quinolines. Mahlo and Eloff (2014) reported the isolation of ursolic acid from the acetone leaf extract of the plant, which displayed vigorous antifungal activity. Nvau et al. (2019) elucidated six other chemical compounds from the stem bark of the plant, while Tlhapi et al. (2021) recently isolated another eight compounds from the same part. The compounds isolated and identified are 7-(β-d-apiofuranosyl-(1→6)-β-d-glucopyranosyl)-umbelliferone and is called adicardin, which is a two-sugar substituted coumarin that readily occurs in plants.

Table 1. Phytochemical screening of the extracts of *Breonada salicina*.

Constituents	Hexane leaf extract	Hexane stem extract	Ethyl Acetate leaf extract	Ethyl Acetate stem extract	Methanol leaf extract	Methanol stem extract
Alkaloids	++	+++	+	+++	++	+++
Saponins	-	-	-	++	+	+++
Tannins	+++	++	+++	+	+++	++
Flavonoids	+	+++	++	+++	+++	+++
Carbohydrates	-	-	-	-	+	+
phenol	+++	++	+++	++	+++	++
Steroids	-	+++	-	++	++	+++
Anthraquinones	+	+++	-	+	++	+++
Cardiac glycosides	-	+++	-	+++	+	++
Terpenoids	++	+++	+++	+++	-	-
Percentage yield	2.07%	2.67%	2.92%	0.85%	16.11%	10.31%

Key: Hex= Hexane Extracts, EA = Ethyl acetate Extracts, MeoH=Methanol; Extracts. -= absent + =present, ++ = More Present, +++ = Highly Present.

The results of the probit analysis for the lethal concentration for the mortality rate of *D. melanogaster* exposed to different extracts of *B. salicina* showed that n-Hexane leaves extract was the most lethal to the flies

with LC_{50} of 38.89 mg/ml, followed by ethyl acetate leave extract 70.39 mg/ml, ethyl acetate stem extract 88.75 mg/ml, methanol leaves extract 215.27 mg/ml, n-Hexane stems extract 231.58 mg/ml and the methanol stem Extract had the lowest toxicity with LC_{50} of 313.01 mg/ml respectively (Table 2).

The percentage scavenging activity of the different leave extracts showed a significant difference (P≤0.05), with methanol extract having the highest scavenging activity in a concentration-dependent manner of between 51.36±0.05 to 83.34±0.10 % which competes favorably with standard antioxidant Ascorbic acid with the scavenging activity of between 58.55±0.01 to 84.35±0.02 %, followed by ethyl acetate with the scavenging activity of between 31.08±0.11 to 66.20±0.02 % and n-Hexane had the lowest scavenging activity of 24.28±0.05 to 57.33±0.03 % respectively (Figure 1). Similarly, the stem extracts also showed scavenging activity with the n-Hexane having the highest activity of between 65.19±0.02 to 84.37±0.00 %, which was not significantly different from the standard antioxidant with the scavenging activity of between 58.55±0.01 to 84.35±0.02 %, followed by the methanol extract with scavenging activity of between 50.47±0.02 to 83.62±0.02 % and ethyl acetate had the lowest scavenging activity of between 42.96±0.05 to 66.63±0.05 % (Figure 2).

Table 2. Probit analysis of the lethal concentration of the mortality rate of D. *melanogaster* on different extracts of *Breonada salicina*.

Part	Extracts	Regression equation	Chi square (P > 0.05)	LC 50	LC90	Lower	Upper
Leaves	n-Hexane	Y = 0.4280*x+0.42	12.129	38.39	127.97	1.899	3.003
	Ethyl acetate	Y = 0.2692*x-2.17	0.874	70.39	450.75	1.100	2.078
	Methanol	Y = 0.1441*x+2.41	0.007	215.27	3188.93	0.586	1.603
Stem	n-Hexane	Y = 0.1462*x+2.33	0.815	231.58	3847.34	0.548	1.553
	Ethyl acetate	Y = 0.2469*x+1.83	2.761	88.75	707.69	0.937	1.906
	Methanol	Y = 0.1127*x+4.83	0.503	313.01	12765.09	0.332	1.259

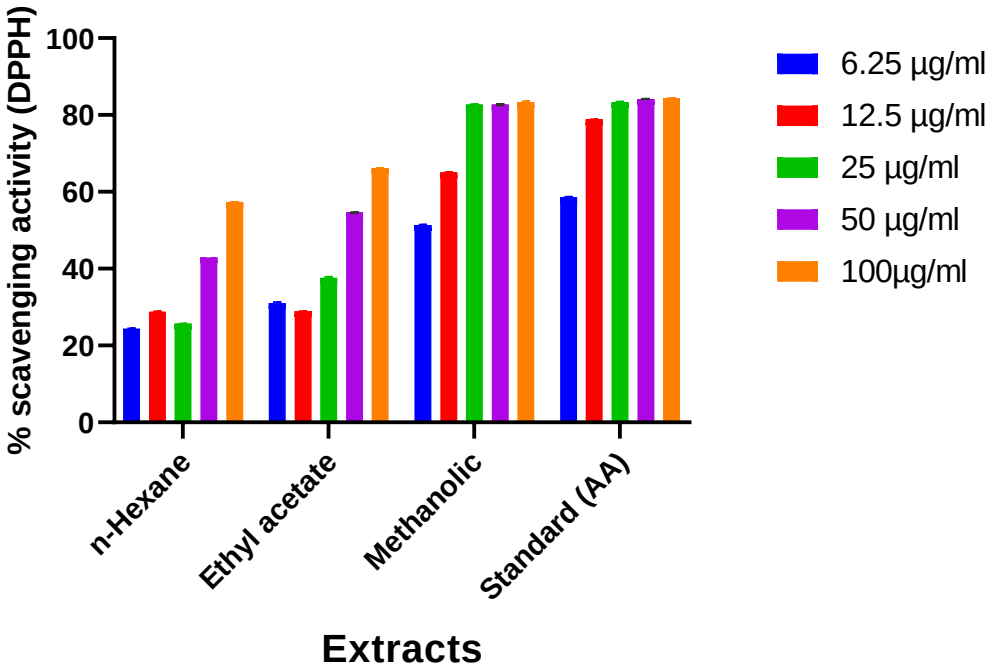

Figure 1. The percentage scavenging activity (SA_{DPPH}) of the different leaf extracts using DPPH radicals.

The results of the biochemical assays revealed that at P≤0.05, there was a significant difference in the biochemical expression of the flies exposed to the LC_{50} of the different extracts (Table 3). In MDA, the n-Hexane leaves and stem had the highest value of between 0.084±0.003 and 0.075±0.003 nmol/mg/protein of flies respectively, followed by the methanol leaves and stem extract with the value of between 0.043±0.001 and 0.062±0.001 nmol/mg/protein and the ethyl acetate had the lowest activity of between 0.020±0.003 and

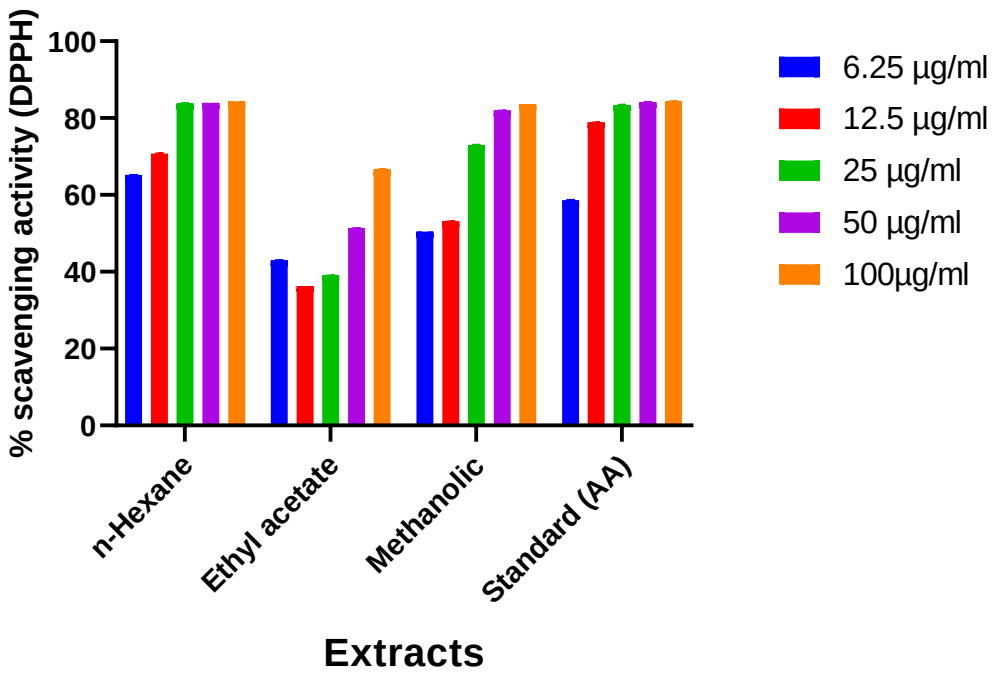

Figure 2. The percentage scavenging activity (SA_{DPPH}) of the different stem extracts using DPPH radicals.

Table 3. The combine biochemical assay of *D. melanogaster* (Harwish strain) exposed to LC 50 of different extracts.

Treatments	**MDA** (nmol/mg/pro.)	**SOD** (µg/mg pro.)	**CAT** (µm/min/mg/pro.)	**GSH** (units /mg/pro.)
n-HexL	0.084± 0.003^{a}	0.922± 0.003^{f}	0.562± 0.004^{c}	30.78± 0.185^{f}
EAL	0.020± 0.004^{e}	2.301± 0.004^{a}	0.351± 0.002^{f}	52.38± 0.06^{e}
MEOHL	0.045± 0.001^{d}	1.371± 0.002^{c}	0.605± 0.002^{b}	601.32±0.10^{a}
n-HexS	0.075± 0.003^{b}	1.181± 0.001^{d}	0.392± 0.003^{e}	30.33± 0.05^{g}
EAS	0.005± 0.001^{f}	1.691± 0.003^{b}	0.221± 0.002^{g}	135.79± 1.51^{c}
MEOHS	0.062± 0.001^{c}	0.971± 0.002^{e}	0.460± 0.002^{d}	54.23± 0.12^{d}
Control	0.003± 0.001^{f}	0.582± 0.002^{g}	1.543± 0.033^{a}	452.66± 2.89^{b}
P-value	<0.0001			
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At P≤0.05 there was a significant difference in the biochemical assay of the *D. melanogaster* (Harwish strain) exposed to LC_{50} of the different extracts. Values are presented as mean standard error of means. Ranking was done across the extracts and values with the same super script are not significant.

0.005±0.001 nmol/mg/protein as compared to the control group which are normal flies on normal diet with the activity of 0.0003±0.001 nmol/mg/protein. Similar results have been reported by other researchers (Chen et al., 2010; Dang et al., 2015; Maag et al., 2015) who reported toxicity of plant extracts in *D. melanogaster*. The consumption of plant extracts by the fly caused toxicity and increased the MDA in *D. melanogaster* (Rzezniczak et al., 2011), leading to a reduction in lifespan, likely due to the damage mediated by reacted oxygen species (ROS) to proteins, lipids and DNA (Finkel and Holbrook, 2000). The toxicity attributed to the test plant could be due to the presence of alkaloids in the extract. Alkaloids are synthesized by many species of plants around the world, and it is known that about half of these compounds are toxic. Plants containing alkaloids are poisonous to livestock and wildlife and have caused tremendous livestock loss (Lucena et al., 2010). Previous studies have shown that D. melanogaster is highly sensitive to the toxic action of alkaloids, leading to genotoxic and mutagenic effects upon exposure to such compounds (Chen et al., 2010).

In SOD activity, the ethyl acetate leave and stem extracts had the highest activity of between 2.301±0.004 and 1.691±0.003 (µg/mg protein) of flies followed by methanol leave extract 1.371±0.002 (µg/mg protein), n-Hexane stems extract 1.181±0.001 (µg/mg protein), methanol stem extract 0.971±0.002 (µg/mg protein) and n-Hexane leave extract had the lowest activity of 0.922±0.003 (µg/mg protein) as compared to the control

group which are normal flies on normal diet with activity of 0.582±0.002 (µg/mg protein). The results of the catalase activity showed that the methanol extract had the highest activity for both the leaves and stem with activities of between 0.605±0.002 and 0.460±0.002 (µm/min/mg/protein) of flies respectively followed by the n-Hexane extract for both leaves and stem with the activity of between 0.562±0.004 and 0.392±0.003 (µm/min/mg/protein) and ethyl acetate had the lowest activity of catalase activity of between 0.351±0.002 and 0.221±0.002 (µm/min/mg/protein) for leaves and stem extract as compared to the control group which are normal flies on normal diet with the activity of 1.543±0.033 (µm/min/mg/protein). The reduced glutathione showed that at P≤0.05 there was a significant difference with the methanol leave extract having the highest activity of 601.32±0.10 (units /mg/protein) of flies than the control group which are normal flies on normal diet with activity of 452.66±2.89 (units /mg/protein), followed by ethyl acetate stem 135.79±1.51 (units /mg/protein), methanol stem 54.23±0.12 (units /mg/protein), ethyl acetate leaves 52.28±0.06 (units /mg/protein) followed by n-Hexane leaves extracts 30.78±0.18 (units /mg/protein) which was not significantly different from n-Hexane stem with activity of 30.33±0.05 (units /mg/protein). There was an induction of GST, SOD and CAT, and these increased activities could represent a compensatory response of cells in counteracting the pro-oxidant effect of plant components. Rotenone, a toxic alkaloid, increased ROS generation and toxic aldehydes in flies (Hosamani and Muralidhara, 2013; Valéria Soares de Araújo Pinho et al., 2014).

CONCLUSION

Oxidative stress, characterized by an imbalance between oxidants and antioxidants, disrupts cellular processes and causes molecular damage. *Drosophila melanogaster*, with its genetic similarity to humans, serves as an excellent model for toxicological studies. Research on the effects of *Breonadia salicina* extracts in fruit flies revealed elevated oxidative stress markers, emphasizing the need for caution in using plant compounds. A comprehensive understanding of phytochemical benefits and potential toxicity is essential for safe medicinal practices.

Conflict of interest

The authors declare no conflict of interest regarding the publication of this paper.

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