**Nectandra acutifolia** (Ruiz & Pav.) Mez (Lauraceae)

Reduces Oxidative Stress Induced with Rotenone in

*Drosophila melanogaster*

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**Abstract:** The increase in the population of neurodegenerative diseases has made it necessary to search for new drugs and phytopharmaceuticals for the prevention or treatment of these diseases, which is why the exploration of the potential of the plants present in the coffee zone is of great importance. The objective of this study was to determine the possible neuroprotective effect of the species *Nectandra acutifolia*. The methanolic extract of *Nectandra acutifolia* was obtained through maceration of the aerial part of the leaves. The phytochemical profile was detected by means of thin layer chromatography. The evaluation of the antioxidant capacity was carried out by means of the colorimetric techniques of DPPH• and ABTS•+. On the other hand, the total content of phenols and flavonoids was determined as well as the neuroprotective activity, by means of the negative geotaxis test and biochemical tests. Finally, the methanolic extract of *N. acutifolia* showed a high antioxidant capacity through the two colorimetric methods. The negative geotaxis test revealed that the concentration of 0.1% had a greater locomotor effect in *D. melanogaster*, which was used as a supplement in the feeding of the *Drosophilas*. The results showed that the methanolic extract of this species has neuroprotective activity using this *in vivo* model.

**Key words:** Antioxidant activity, neuroprotection, *Nectandra acutifolia*, antioxidant capacity, locomotive capacity, negative geotaxis.

1. **Introduction**

Natural products have been tested in various animal models for the study of different diseases and their mode of action has been widely explored in order to find new bioactive molecules [1, 2], this is how plants such as *Cannabis sativa, Brassica nigra, Cuscuta reflexa, Withania somnifera* among others have traditionally been used for the treatment of neurodegenerative diseases [3]. This neurological deficit produces several diseases. One of them is Parkinson’s disease (PD). PD is a neurodegenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra of the brain [4].

Animal models such as *D. melanogaster, Caenorhabditis elegans, Danio rerio* and *Mus musculus* [5] have been used for the knowledge of this disease and neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium (MPTP), its ion MPP+, 1,1-dimethyl-4,4-dipyridinium (paraquat), rotenone and 6-hydroxydopamine (6-OHDA) [6] have been used to generate the brain damage and induce the oxidative stress in the mitochondria that ultimately produces damage to dopaminergic neurons and produces the motor symptoms of Parkinson’s disease, which include slow movements, tremor at rest and rigidity.

One of the invaluable models in biology and genetics that has been used for more than 100 years is *Drosophila melanogaster* (*D. melanogaster*) because it is an organism that offers multiple benefits from the
Nectandra acutifolia (Ruiz & Pav.) Mez (Lauraceae) reduces oxidative stress induced with rotenone in Drosophila melanogaster

600 genetic point of view, given that most of the genes that codify for Parkinson’s are homologous to the human ones [7]. In addition, it is easy handling at the laboratory level.

Many extracts of different plant species have presented neuroprotective activity with different models [2, 5] including D. melanogaster. On the other hand, several phytochemical investigations have shown that many species of the genus Nectandra (Lauraceae) have presented a wide source of bioactive molecules [8] mainly related to biological activities as an antioxidant [9]; inhibition of COX-1, COX-2 and LOX-5 [10], vasooactive [11], anti-malaria [12, 13] and anti-mycobacterium tuberculosis [14] and neuroprotective potential [15, 8]. The methanolic extract of Nectandra acutifolia (Ruiz & Pav.) Mez has presented a strong anti-fungal property against Mycosphaerella fijiensis Morelet [16], confirming in this way that this species has a wide range of very interesting large-scale compounds to be evaluated. For this reason, the objective of this research was to evaluate the neuroprotective effect of the methanolic extract of said species with the in vivo model of D. melanogaster, thus contributing to the knowledge of the phytochemical potential contained in the vegetation of the coffee zone.

2. Materials and Methods

2.1 Reagents, Solvents and Equipment

The solvents used were analytical grade and Mallinckrodt brand HPLC (Phillipsburg, NJ, USA), the trolox reagents (6-hydroxy-2,5,7,8-tetramethylchrome-2-carboxylic acid), DPPH’ (2,2-diphenyl-1-picyrylhydrazyl), ABTS”(2,2'-azinobis-(3-ethylbenzthiazoline)-6-ammonium sulfonate acid), Folin & Ciocalteu 2 N reagent and gallic acid (3,4 acid, 5-trihydroxybenzoic acid), silica gel chromatographs F254 0.2 mm were Merck (Darmstadt, Germany). For all the determinations, a Multiskan GO/UV microplate spectrophotometer Thermo Scientific (Vantaa, Finland) was used. The rotenone was acquired at Sigma-Aldrich (St. Louis, MO, USA). A Fluoroskan Ascent Thermo Scientific (Vantaa, Finland) was used to determine the lipid superoxidation.

2.2 Preparation of the Plant Extract

The plant was collected in the Bremen-La Popa Natural Reserve in 2006 by the taxonomist of the University of Antioquia Francisco Javier Roldan and the voucher assigned for this species was FJR 4032. The aerial part of the plant was dried at 50 °C, later it was ground and extraction was made by passive maceration following the methodology described by Mosquera et al. [17]. First the extraction was carried out with hexane, then with dichloromethane (DCM) and finally with methanol. The extracts were stored at -10 °C until use.

2.3 Determination of Phytochemicals by Thin Layer Chromatography (TLC)

The methanolic extract was characterized by thin layer chromatography following the protocol of Wagner and Bladt [18]. Silica gel 60 F254 (5 × 4 cm) chromatography was used, the extract was eluted with the ethyl acetate system (AcOEt); ethanol; DCM (60:10:30).

2.4 Determination of the Content of Total Phenols

For the determination of the content of phenols, we followed the methodology described by Magalhães et al. [19], in which 50 μL of the Folin-Ciocalteu reagent (1:50, v/v) was added to 50 μL of the 100 mg/L extract and immediately afterwards 100 μL of a NaOH solution (0.35 M) was added. This mixture was allowed to react for three minutes and the absorbance at 760 nm was measured. For this determination, a calibration curve for gallic acid was made at 0, 2, 4, 8 and 16 μg/gallic acid/mL.

2.5 Determination of the Total Flavonoid Content

For the determination of the total flavonoid content, the methodology described by Kim et al. [20] was
fitted. The mixture consisting of 20 μL of the extract at 50 mg/L plus 115 μL of H2O was prepared and 7.5 μL of NaNO2 (5%) was added. It was allowed to react for 5 minutes and subsequently 30 μL of AlCl3 (2.5%) was added, allowing this reaction for 6 minutes. After this period 50 μL of NaOH (1 M) and immediately 50 μL of H2O were added; after 5 minutes, the absorbance at 500 nm was measured. For the quantification of total flavonoids, a calibration curve of 0.05, 0.1, 0.2, 0.4 and 0.8 μg kaempferol/mL was performed.

2.6 Determination of Antioxidant Capacity by the DPPH’ Method

Antioxidant capacity was measured through the DPPH radical method described by Brand-Williams et al. [21] with modifications. The antioxidant capacity was determined by reacting 100 μL of a solution of DPPH’ at 20 mg/L and 25 μL of a solution of the extract at 1000 mg/L for half an hour under dark conditions and at room temperature (A sample). As a negative control, the solvent in which the extracts were solubilized (A control (-)) was used and as a positive control hydroquinone at 1000 mg/L was used. The reduction of the radical was measured at λ = 517 nm. The percentage of discoloration of DPPH’, was determined by the following equation:

\[
\text{% Antioxidant activity} = \left( \frac{A\ \text{control} (-) - A\ \text{sample}}{A\ \text{control} (-)} \right) \times 100
\]

A calibration curve of trolox was carried out at the concentrations of 1, 2, 4, 8, 16, 32 and 64 μM to express the antioxidant capacity in trolox equivalents (μmolTrolox/g extract).

2.7 Determination of the Antioxidant Capacity by the ABTS’+ Method

The ability to inhibit the radical 2,2’-azino-bis (3-ethylbenzthiazoline)-6-ammonium sulphonate (ABTS’+) by the extracts studied was determined following the method proposed by Re et al. [22]. The radical was generated by the oxidation of ABTS’+ at 3.5 mM with potassium persulfate at 1.25 mM. To carry out the test, 6 μL of the extract was taken at 1000 mg/L and mixed with 294 μL of ABTS’+ solution previously prepared and adjusted to an absorbance value of 0.700 ± 0.002 with ethanol. This mixture was allowed to react for half an hour in the absence of light and at room temperature. The reduction of the ABTS’+ was measured at λ = 732 nm. The negative and positive controls were the same as those used in the DPPH’ test as well as the equation used for the determination of the antioxidant activity. A calibration curve of trolox was carried out at the concentrations of 1, 5, 10, 20, 40, 80 and 100 μM to express the antioxidant capacity in trolox equivalents (μmolTrolox/g extract).

2.8 Culture Conditions of D. melanogaster, Exposure to Rotenone and Treatments

D. melanogaster (wild type), is donated by the University of Caldas, Manizales, Colombia. Keep it at an ambient temperature of 24-25 °C. The standard medium was composed of 1.19 g/mL of banana, 1.2% m/v of agar, 1% m/v of yeast, 0.75% v/v of micostatin and 0.18% v/v of propionic acid. For all studies the age of D. melanogaster was synchronized for 8-12 days and males were used.

The males of D. melanogaster from 1-8 days of age were divided into six groups of 45 flies each: (1) coexposure, extract + rotenone, for 14 days; (2) 1. rotenone for 7 days, then extract for 7 days; (3) 1. extract for 7 days, then rotenone for 7 days; (4) extract, for 14 days; (5) control and (6) rotenone. The rotenone used was diluted in ethanol and the methanol extract of T. cf discolor was diluted in distilled water, the concentration used was 0.1%.

The flies were exposed to four treatments for a period of 14 days (45 flies in total for each treatment, with its respective repetition), in addition to control flies and flies exposed to rotenone. The treatments are mentioned below: (treatment 1 = coexposure, extract + rotenone, for 14 days); (treatment 2 = 1. rotenone for 7 days, then extract for 7 days); (treatment 3 = 1. extract for 7 days, then rotenone for 7 days); (treatment 4 = extract, for 14 days); (treatment 5 = control and (6 = rotenone. The rotenone used was diluted in ethanol and the methanol extract of T. cf discolor was diluted in distilled water, the concentration used was 0.1%.

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for 7 days, then rotenone for 7 days); (treatment 4 = extract, for 14 days). The concentration of the extract used was 0.1% m/v. Table 1 shows the scheme of the treatments.

2.9 In Vivo Tests

2.9.1 Negative Geotaxis

The methodology described by Sudati et al. [23] was used to determine locomotion patterns. The flies were kept in banana-based food, then the separation by gender was carried out, selecting only the males for the experiment. They were exposed to different concentrations of the methanolic extract of *Nectandra acutifolia* (0.05, 0.1 and 0.2%). Once the concentration that did not induce any damage to the motor response of *D. melanogaster* was found, it was co-exposed to extract and rotenone (0.1% methanolic extract). The motor function was evaluated by means of the negative geotaxis test and the locomotor behavior was expressed with the number of flies reaching a minimum distance of 6 centimeters in 6 seconds. Finally, the results were analyzed with the GraphPad PRISM 5.0 software.

2.9.2 Longevity Test

The effect of the methanol extract of *T. cf discolor* on the stressed flies with rotenone (100 μM) was evaluated, using the methodology of Rao et al. [24]. Male flies 3-5 days old were kept in the medium enriched with treatment 1, treatment 4, control and rotenone. The flies were changed to another medium every three days, until the last fly died.

2.10 Ex Vivo Tests

2.10.1 Preparation of Heads Homogenization

Forty-five (45) flies were anesthetized with diethyl ether for approximately 1 minute. Subsequently, the heads were homogenized in 450 μL of phosphate buffer, pH 7.4. Then, the homogenate was centrifuged at 1000 rpm for 10 minutes at 4 °C; the supernatant was removed and used for biochemical tests.

2.10.2 Protein Determination

The protein concentration was measured by the Bradford method [25], using bovine serum albumin as standard.

2.10.3 Determination of Lipid Superoxidation

The protocol of Ohkawa et al. was used [26]. Using the Cayman Chemical Kit, the procedure is described below: 25 μL of the sample or standard was added in an eppendorf. Subsequently, 25 μL of sodium dodecyl sulfate (SDS) and 1 mL of the color reagent composed of tiobarbituric tiobarbituric acid (TBA), acetic acid and NaOH were added. The eppendorf was taken to the thermostatic bath at 95 °C for 1 hour, after that the samples were left in a bath with ice for 10 minutes and finally centrifuged at 1000 rpm at 4 °C. Then, 150 μL of the sample was placed in a well of a microplate and the fluorescence was read at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.10.4 Activity of Antioxidant Enzymes

**Activity of the Catalase (CAT).** It was determined by the Aebi protocol [27]. The reaction mixture was composed of 8.8 mM H₂O₂ and 0.1 mM sodium phosphate buffer, pH 7.

The reaction was started by adding the homogenate of *D. melanogaster*. The decrease of the H₂O₂ was monitored for 3 minutes at 240 nm.

**Activity of Superoxide Dismutase (SOD).** The methodology described by Yang et al. [28] was used. To determine the activity of mitochondrial superoxide

| Table 1 | Treatments applied to *D. melanogaster.* |
|---------|-----------------------------------------|
| Treatment 1 | Rotenone + Extract (R + E) |
| Treatment 2 | 1-Rotenone-2-Extract (1-R-2-E) |
| Treatment 3 | 1-Extract-2-Rotenone (1-E-2-R) |
| Treatment 4 | Extract (E) |
| Treatment 5 | Control (C) |
| Treatment 6 | Rotenone (R) |
Nectandra acutifolia (Ruiz & Pav.) Mez (Lauraceae) Reduces Oxidative Stress Induced with Rotenone in Drosophila melanogaster

Dismutase, the following reagents were added in a vial: 150 μL of KCN phosphate buffer, 5 μL of the homogenate, 40 μL of deionized H2O, 15 μL of xanthine, 15 μL of hydrochlorinated hydroxylamine and 75 μL of xanthine oxidase. The above mixture was allowed to react in a dark place at room temperature for 20 minutes and the reaction was stopped with an ice bath. Then 50 μL of sulphamic acid and 50 μL of α-naphthylamine are added in the well of a microplate. Subsequently, 50 μL of the initially prepared mixture is added to the well containing the sulphanic acid and the α-naphthylamine. The new mixture is incubated for 20 minutes at room temperature and the absorbance at 529 nm is read.

Acetylcholinesterase (AChE) Activity. The method described by Nair et al. [29] based on the Ellman method was used. The following reagents were prepared, solution A (Tris HCl, 50 mM); solution B (Tris HCl, 50 mM with 0.1 of ASB); solution C (3 mM DTNB in 50 mM Tris HCl buffer); solution D (Acetyl choline iodide (IATC) 15 mM). Transfer 25 μL of the extract to each well, and then add 125 μL of solution C, then 50 μL of solution B and finally 25 μL of AChE 0.3 U/mL solution. It is incubated for 15 minutes at 37 °C and after this time the absorbance at 405 nm is read.

Statistical Analysis. Initially descriptive statistics such as the standard deviation (SD) were made. They were performed for each of the three measurements independently. The statistical analysis was performed in the GraphPad Prism version 5 software. The student t-test was performed and one-way analysis with Tukey post-test was performed to determine if the differences between treatments and controls were significant with p < 0.05.

3. Results

By means of thin-layer chromatography, the nuclei shown in Table 2 were found. An EC50 50.63 μg/mL was determined by the DPPH• method. The EC50 for ascorbic acid was 3.65 μg/mL and for hydroquinone it was 3.34 μg/mL. This species presented a content of 0.238 ug of gallic acid/ug of extract of total phenols and a content of total flavonoids of 1.443 ug of quercetin/ug of extract (Table 3).

The results obtained to select the concentration to be evaluated are presented in Fig. 1, and none of the concentrations evaluated showed significant differences compared to the control. The selected concentration was 0.1%.

To determine the concentration of the extract to be used in the subsequent treatments, the flies were exposed for a period of 21 days at the selected concentration and

| Phytochemical core | Presence |
|-------------------|----------|
| Phenols           | ++       |
| Flavonoids        | ++       |
| Alkaloids         | +        |
| Lactones          | -        |
| Anthraquinones    | -        |
| Terpenes          | +        |
| Coumarinas        | ++       |
| Saponins          | ++       |
| Steroids          | -        |

(+): Presence of the nucleus; (++): intermediate presence of the nucleus; (-): absence of the nucleus.

| Table 3  Content of total phenols, total flavonoids and antioxidant activity. |
|-----------------------------|------------------|
| Content of phenols (μg of gallic acid/μg of extract) | 0.2387 |
| Flavonoid content (μg of quercetin/μg of extract)    | 1.4429 |
| EC50 (μg/mL) DPPH•                                       | 50.63  |
Nectandra acutifolia (Ruiz & Pav.) Mez (Lauraceae) Reduces Oxidative Stress Induced with Rotenone in Drosophila melanogaster

Fig. 1  Number of flies that rise 6 cm/6 seconds after being exposed 7 days to different concentrations of the extract of N. acutifolia. Values are the averages of two tests with three replicas for each. Data analyzed by one-way ANOVA (p < 0.05), followed by Tukey’s multiple comparisons test.

Fig. 2  Results of the negative geotaxis test. Number of flies that rise 6 cm/6 seconds after being exposed 21 days to the extract and to the rotenone. Values are the averages of two tests with three replicas for each (* significance with respect to rotenone (p < 0.05)).

it was determined that the extract of N. acutifolia at the concentration of 0.1% did not show any damage to the locomotor system, compared with rotenone. The data are presented in Fig. 2.

Fig. 3 shows the results of the longevity test, the exposure of the flies to treatment 1 (R + E), increased the life time by 17 days compared to rotenone, with which they only lived 55 days. The flies of treatment 4—only extract (E), lived 98 days and the control flies lived 97 days.
Fig. 4 shows that all treatments presented differences compared to rotenone and none of the treatments presented differences in relation to the control in the negative geotaxis test.

Fig. 5a shows a significant increase in the malondialdehyde level of the flies exposed to rotenone compared to the control ones \((p < 0.05)\). All the other treatments presented significant differences in relation to rotenone.

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**Fig. 3** Effect of treatments on the survival of flies. The values are the survival percentages \((n = 45\) per treatment). The test was performed in duplicate, with three replicas for each.

**Fig. 4** Number of flies that rise 6 cm/6 seconds after being exposed 14 days to the different treatments. Values are the averages of two tests with three replicas for each. Data analyzed by one-way ANOVA \((p < 0.05)\), followed by Tukey’s multiple comparisons test (* significance with respect to rotenone \((p < 0.05)\)).
to rotenone ($p < 0.05$) and only the treatment with the extract presented differences in relation to the control ($p < 0.05$). Interestingly, the treatment with the extract alone decreased the level of malondialdehyde, even less than the control.

Fig. 5b shows that no significant differences in SOD activity were observed between control flies and rotenone-treated flies, but the other treatments did show significant differences ($p < 0.05$) compared with rotenone. The treatments R + E and extract only increased the activity of the SOD and presented significant differences compared to the control ($p < 0.05$).

In Fig. 5c (determination of catalase activity), the treatments R + E, 1-E-2-R and only extract E, presented significant differences compared to the control ($p < 0.05$). The treatments 1-R-2-E, 1-E-2-R, E and C presented differences compared to rotenone ($p < 0.05$).

In Fig. 5d (determination of the activity of acetylcholinesterase), all the treatments presented significant differences in comparison with rotenone. The treatments R + E and 1-R-2-E, presented differences with respect to the control.

![Graphs showing malondialdehyde (MDA), SOD activity, catalase activity, and acetylcholinesterase activity.](image)

**Fig. 5** (a) Quantification of malondialdehyde (MDA); (b) activity of dismutase superoxide; (c) catalase activity and (d) acetylcholinesterase activity. Values are the averages of two tests with three replicas for each. Data analyzed by one-way ANOVA ($p < 0.05$), followed by Tukey’s multiple comparison test (* significance with respect to rotenone; + significance with respect to control).
4. Discussion

The results obtained in this investigation using *D. melanogaster* as a study model, showed that the methanolic extract of *N. acutifolia* (Ruiz & Pav.) Mez, allowed the locomotor capacity of the *Drosophilas* to improve after having been exposed to rotenone. In the negative geotaxis test performed after the *Drosophilas* were exposed to the different treatments, it was determined that the treatment of rotenone decreased the number of flies that ascend through the geotaxic tube, which indicates the damage caused by this neurotoxic substance.

Rotenone increases oxidative stress in cells and this increase in reactive species is involved in the development of various neurodegenerative diseases, the mechanism by which oxidative stress participates in the death of dopaminergic neurons is not yet very clear, but plays a very important role [30].

One of the indicators of the damage caused by oxidative stress is the content of malondialdehyde in the cell. In addition, the endogenous antioxidant system of an organism can also give indications of the damages caused by the reactive oxygen species, as it is the case of the enzymes superoxide dismutase and catalase, which are responsible for defending the cell, a low or high activity of them, and indicate the state of the cell, studies conducted by Milani et al. [31] and Bottle et al. [32] demonstrated that overexpression of SOD protects dopaminergic neurons.

Many authors have reported that several plant extracts with a high antioxidant potential have presented neuroprotective activity with *D. melanogaster* [33, 34].

![Possible mechanism of protection of the methanolic extract of *N. acutifolia*.](source)

Source: prepared by the authors.
The reduction of oxidative stress attributed to the extract of *N. acutifolia*, is mainly due to its high antioxidant capacity as evidenced by the two methods evaluated in this study and by the high content of phenols and flavonoids, for which this type of compounds would be responsible for the neutralization of the free radicals. In addition, the exposure of *Drosophilas* to the extract reduced the levels of malondialdehyde and increased the activity of SOD and catalase.

Additionally, in the longevity test it was determined that the extract of *N. acutifolia* also extended the life time of the *Drosophilas*, which agrees with the study carried out by Liu et al., where the extract of *Panax ginseng* extended the life time of *D. melanogaster* and increased SOD activity and decreased MDA levels [35].

With the results obtained in this study, the possible mechanism of protection of the extract of *N. acutifolia* was presented, as shown in Fig. 6. This protection of the extract could be attributed to the phenolic compounds and flavonoids that are present in the mixture and that were detected by thin layer chromatography and quantified, where a high content of phenolic compounds was found in the extract or perhaps in the synergy of all the components of the extract.

On the other hand, Reglodi et al. [36] have mentioned that polyphenols, antibiotics and neuropeptides are the new candidates for future treatments for Parkinson’s disease. In addition, the results obtained in this research have shown that the extract of *N. acutifolia* has a great potential as a neuroprotector, so it would be interesting in future research to perform a fractionation to determine which would be the phytochemical nuclei responsible for the neuroprotective activity or if the compounds present in the extract act synergistically.

5. Conclusions

This is the first report to state that the methanolic extract of *N. acutifolia* has a high content of phenols and flavonoids, which is why its high antioxidant capacity could be attributed to these compounds. In addition, this extract also showed neuroprotective capacity, so in future investigations it would be interesting to isolate and identify its constituents with neuroprotective capacity to prevent neurodegenerative disorders.

Acknowledgements

To the vice-rectory of Research, Innovation and Extension of the Technological University of Pereira and to the project: Development of Scientific and Technological Capacities in Biotechnology Applied to the Health and Agro-Industry Sectors in the Department of Risaralda (code: BPIN 201200010050) financed by the General System of Royalties.

Conflicts of Interest

The authors do not present a conflict of interest.

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Nectandra acutifolia (Ruiz & Pav.) Mez (Lauraceae) Reduces Oxidative Stress Induced with Rotenone in Drosophila melanogaster

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