Comparative Neuroprotective Activities of the Ethanolic extracts of the Roots, Barks, Seeds, and Fruitless Bunches of Washingtonia filifera

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ABSTRACT

The present study aimed to the in vitro investigation of the ethanol extracts of Washingtonia filifera roots (W.R), barks (W.B), leaves (W.L), fruitless bunches (W.F) and seeds (W.S) for their neuroprotective activities including the phenolic content, antioxidant activity, anti-inflammatory activity and the potentiality to protect mouse neuroblastoma (neuro-2A) cells against H2O2 and Aβ1-42-induced neurotoxicity. Among the extracts, W.L and W.F had the highest phenolic content; 226.7 and 172.3 GAE, respectively. Consequently, W.L and W.F showed pronounced DPPH radical scavenging activity with IC50 values of 19.95 and 30.46 µg/mL, respectively. The highest inhibitory potential of COX-2 was achieved by W.F then W.L with IC50 values of 111.62 and 191.77 µg/mL. The most efficient extract for the neuroprotection against H2O2 and Aβ1-42 -induced neurotoxicity was W.F which had more potent activity than the positive control in Aβ1-42 assay. To the best of our knowledge, this is the first report to indicate the neuroprotective activities of W. filifera leaf and the fruitless bunch extracts.

Keywords: W. filifera; neuroprotection; H2O2; Aβ1-42; anti-inflammatory.
1. INTRODUCTION

Washingtonia is a palm genus of two species W. filifera and W. robusta. It belongs to the family Arecaceae that includes approximately 2500 species. W. filifera (L. Linden) H. Wendl "commonly called California palm or desert fan palm" is an endemic species and the only native ornamental palm to the western United States. However, it had been introduced to Egypt and elsewhere. For decades, the fan palm fruits were eaten for their sweet taste and healthy values. The basal tissues of the leaves were reported to have larvicidal activity against red palm weevil due to chalconoid analogue filiferol compound. Leaves and fruits were proven to have antiviral activity against coxsackievirus B3. Proanthocyanidins with antioxidant, anti-butyrylcholinesterase, and anti-xanthine oxidase were reported from the seeds. Furthermore, the seed extracts exhibited inhibition of amyloid polypeptides aggregation, α-amylase and α-glucosidase.

Leaves and flowers exhibited antioxidant activity which was attributed to their composition of tricine acetylated derivative and heterocyclic bioactive Quinazoline-2, 4-(1H, 3H) dione. Aerial parts and leaves were reported to have powerful antioxidants flavonoids such as luteolin 7-O-glucoside 4'-sulfate and 8-hydroxyisoscoparin, together with vitexin, isovitexin7-O-methyl ether, luteolin 7-O-glucoside, luteolin7-O-β-D-glucoside -2'-sulfate, luteolin7-O-β-D-glucoside-4'-sulfate, orientin, iso-orientin, 8-hydroxyisocoparin, tricin 7-O-β-D-glucoside and tricin7-rhamnopyranoside (1'' 6''). W. filifera has a yearly huge amount of waste in the form of dried fruit bunches, barks, leaves, fruits, and seeds. Most of W. filifera waste hasn’t been investigated for its biological activities.

The complications of neurodegenerative diseases (ND) such as Alzheimer’s disease (AD) and Parkinsonism are the riskiest factors in elderly populations. They are primarily characterized by increased oxidative stress and neuroinflammation which consequently lead to neuronal death, loss of cognitive and motor functions. The recent treatments of ND are mostly symptomatic, so there is an urgent demand for searching for new protectives and therapies, especially from natural resources. Also, there are risk factors; in the presence of the metal components of the cell medium, H2O2 is converted into hydroxyl radicals which increase the oxidative stress and stimulate the cascade of events that may result in neuronal cell death. So, the antioxidant extracts will be able to scavenge the free radicals resulting in increasing cell viability.

To the best of our knowledge, there was no previous report about the neuroprotective activities of W. filifera parts other than the seeds. So, this study includes the ethanol extracts of the fruitless bunches (WF), roots (WR), barks (WB), leaves (WL), and seeds (WS) of W. filifera were comparatively screened for their in vitro antioxidant, anti-inflammatory and neuroprotective activities to pave the way for their phytochemical screening in the direction of valuing potential neuroprotective leads from medicinal plants.

2. METHODS

2.1. Plant materials

Roots, barks, leaves, seeds, and fruitless bunches of W. filifera were collected from the garden of the Faculty of Pharmacy, Mansoura University on 11th November 2022 and were identified by Associate Prof. Dr. Mahmoud Makram Qassem, Department of Vegetables & Floriculture, Faculty of Agriculture, Mansoura University, Egypt where a voucher specimen (Voucher No.WF-07) was deposited at the department of pharmacognosy, Mansoura University. Plant materials were then washed with water to get rid of dirt and were shade-dried for several days. The dried material was ground into a coarse powder and stored at a refrigerated temperature until use.

2.2. General

Hydrogen peroxide (H2O2). Aβ1-42 stock solution: Aβ1-42, cyclooxygenase-2 (COX-2) Cayman human enzyme inhibitory assay kit (No. 701070), COX-2 Cayman human enzyme inhibitory assay kit (No.701080, USA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid celereoxib®, galantamine, catechin, epigallocatechin-3-gallate were purchased from Sigma Chemical Co (Sigma Aldrich St Louis, MO, USA). Mouse neuroblastoma (neuro-2A) cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

2.3. Preparation of plant material

Two hundred grams of each of the fruitless bunches, roots, barks, leaves, and seeds of W. filifera palms were separately soaked in ethanol (400 mL x 3) at room temperature (25°C) in amber-colored extraction bottles. The extracts were separately filtered and concentrated using the rotary evaporator under reduced pressure at 50°C to afford 10% (5%), 17.5 (8.75%), 15 (7.5%), 63 (31.5%), and 17 (8.5%) g extract of each of W.F, W.R, W.B, W.L, and W.S, respectively.

2.4. Determination of total phenolic content

The total phenolic contents were measured in the extracts as previously described. Briefly, 0.5 mL of each sample at a concentration of 2 mg/mL or ethanol (as a negative control) was mixed with 1 mL of 10% of Folin-Ciocalteu reagent and 4 ml of 700 mM sodium carbonate. This mixture was shaken and allowed to stand for 2h. The reaction color was measured spectrophotometrically at 765 nm. Gallic acid was used to prepare the standard curve and phenolic
2.5. DPPH-assay

The ability of the extracts to scavenge DPPH radical was spectrophotometrically measured at 517 nm \(^{16}\). The assay was done as previously described \(^{17}\). The antioxidant activity (AA) was measured as follows:

\[
AA\% = \frac{(A_{\text{blank}} - A_{\text{sample}} - A_{\text{control}})}{A_{\text{blank}}} \times 100
\]

IC\(_{50}\) was calculated from the regression equations of the curves by plotting the % inhibition against sample concentrations.

2.6. In vitro COX-2 inhibitory assay

The Procedures were carried out according to the manufacturer’s protocol using a COX-2 Cayman human enzyme inhibitory assay kit (No.701080, USA), ROBONIK P2000 EIA reader, and Celecoxib® as a positive control \(^{18}\). Four Parameter Logistic Curve online data analysis tool of My Assays Ltd was used to evaluate the data.

2.7. In vitro neuroprotective activity

2.7.1. Cytotoxic activity of the extracts on neuro-2 A cells

Neuro-2A cells (2 x 10^4 cells/ well) were incubated in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 20 mm glutamine, 10 U/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Gaithersburg, MD, USA) with different concentrations of the extracts (25, 50, 100 and 200 μg/ mL). After 48 h, cell viability was determined using the WST-1 reagent (Sigma Aldrich St Louis, MO, USA) as previously described \(^{19}\).

2.7.2. Effect of the extracts on cell viability of neuro-2A cells after treatment with H\(_2\)O\(_2\) as a neurotoxic agent

In this experiment, the concentration of H\(_2\)O\(_2\) that could decrease cell viability to about 50% was determined as previously described \(^{20}\). The results showed that H\(_2\)O\(_2\) at a concentration of 150 μM had the desired decrease in cell viability (Figure 2A). Cells (2 x 10^4 cells/ well) were incubated with different non-cytotoxic concentrations (6.25, 12.5, 25, 50, 100 μg/mL) of the tested extracts or catechin as a positive control \(^{21}\). After two hours the media were replaced with fresh ones and the cells were treated with H\(_2\)O\(_2\) (150 μM) for 24 h. Finally, cell viability was determined using WST-1 reagent.

2.7.3. Effect of the extracts on cell viability of neuro-2A cells after treatment with Aβ1-42 as a neurotoxic agent

Aβ1-42 Stock solution (1 mM) was prepared by dissolving Aβ1-42 peptide in deionized distilled water and incubated for 3 days at 37°C, then it was kept at -20°C. Aβ1-42 concentration that caused about 50% suppression of cell viability was detected. Briefly, 96 well plates were seeded by neuro-2A cells at a cell density of 1x10^4 cells/ well. After 48 h different concentrations of Aβ1-42 (12.5, 25, 50 μM) were separately added and incubated with the cells for another 24 h. Cell viability was determined using a WST-1 reagent. Aβ1-42 at 25 μM resulted in the desired reduction of cell viability (Figure 3A) and was used for the determination of the neuroprotective activities of the tested compounds. The cells were treated with different concentrations of the tested samples or epicatechin-3-gallate as a positive control \(^{22}\) (6.25, 12.5, 25, 50, 100 μg/mL) or DMSO as a negative control. After 24 h the media were replaced with fresh one and the cells were incubated for 48 h with 25 μM of Aβ1-42. Finally, cell viability was determined using WST-1 reagent.

2.8. Statistical analysis

All experiments were carried out in independent triplicates. Figures were built in Microsoft Excel 2010. All values were the mean ± SD. Statistical significance was determined using one-way ANOVA followed by Dunnett’s post hoc test in GraphPad Prism\(^{10}\) (Version 10.0.3, GraphPad Software, Inc., USA).

3. RESULTS AND DISCUSSION

3.1. Determination of the total phenolic content

The ethanol extract of the leaves (W. L) showed the highest amount of phenolic content (226.7 GAE) while that of the bark (W.B) had the lowest amount (99.97 GAE). The ethanol extract of the fruitless bunches (W.F) had higher phenolic content than that of roots (W.R) and seeds (W.S) by 54.4 GAE (Table 1).

The high phenolic content of (W. L) might be due to its flavonoid compounds; luteolin, tricin \(^{23}\) and 5,7,4ˈ - triacetate tricin \(^{8}\). In addition, a polyhydroxy chalconoid analogue; filiferol was previously isolated from the basal tissues of the leaves \(^{5}\).

3.2. Determination of the antioxidant activity using DPPH assay

The results of the DPPH assay manifested good matching with the phenolic contents of the ethanol extracts of different parts of W. filifera. All the extracts showed IC\(_{50}\) values for the scavenging of the DPPH free radicals less than...
100 µg/mL (Table 1). The antioxidant activity was achieved in the following order: W.L > W.F > W.R > W.B > W.S.

The antioxidant potential of only leaves, aerial parts, and seeds was previously studied. The antioxidant activity of different extracts of the leaves and aerial parts was formerly evaluated using DPPH \(^a\) and \(\beta\)-carotene bleaching method \(^10\). It was found that the ethanol extract of the leaves and the ether extract of the aerial parts had the highest antioxidant activities. The antioxidant activity was justified by the presence of flavonoid compounds; vitexin, isovitexin\(7\)-\(O\)-methyl ether, luteolin \(7\)-O-glucoside, luteolin\(7\)-\(O\)-\(\beta\)-D-glucoside \(-2\)\(^\prime\)-sulfate, luteolin\(7\)-\(O\)-\(\beta\)-D-glucoside\(-4\)\(^\prime\)-sulfate, orientin, iso-orientin, 8-hydroxyisoscoparin, tricin \(7\)-\(O\)-\(\beta\)-D-glucoside and tricin\(7\)-rhamnopyranoside \((1'' \rightarrow 6'')\) glucopyranoside \(^10\). On the contrary, hexane extract of mature and immature seed was previously found to be inactive in ABTS radical scavenging assay \(^24\).

### 3.3. Determination of the in vitro anti-inflammatory activity

The anti-inflammatory activity of the extracts was determined by measuring their efficiency in inhibiting the COX-2 enzyme that is responsible for the conversion of arachidonic acid to prostaglandins which are the main controller of pain sensation and the inflammatory process \(^25\).

The ethanol extract of the fruitless bunches (W.F) showed the strongest in vitro anti-inflammatory activity (lowest IC\(50\) value) through the inhibition of the COX-2 enzyme. W.L and W.B had less potent activities while W.R and W.S couldn’t inhibit COX-2 enzyme at concentrations up to 1 mg/mL (Table 1).

Table 1: Phenolic content and IC\(50\) values of different ethanol extracts of \(W.\ filifera\) in DPPH and COX-2 assays

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC(50) values (µg/mL)</th>
<th>Phenolic content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH(^a)</td>
<td>COX-2(^b)</td>
</tr>
<tr>
<td>W.R</td>
<td>55.12 ± 0.21</td>
<td>&gt; 400</td>
</tr>
<tr>
<td>W.B</td>
<td>67.63 ± 0.32</td>
<td>320.28 ± 9.87</td>
</tr>
<tr>
<td>W.L</td>
<td>19.95 ± 0.12</td>
<td>191.77 ± 8.21</td>
</tr>
<tr>
<td>W.F</td>
<td>30.46 ± 0.14</td>
<td>111.62 ± 5.43</td>
</tr>
<tr>
<td>W.S</td>
<td>80.83 ± 0.43</td>
<td>&gt; 400</td>
</tr>
</tbody>
</table>

\(^a\) Ascorbic acid was used as a positive control and had IC\(50\) = 17 ± 0.1 µg/mL.

\(^b\) Celecoxib\(^b\) was used as a positive control and had IC\(50\) = 13.13 ± 0.49 µg/mL.

\(^c\) expressed in terms of GA\(E\).

The anti-inflammatory activity of the leaves (W.L) might be due to the presence of several compounds such as vitexin which previously showed significant in vivo reduction of COX-2 levels \(^26\), orientin which was proved to decrease the expression of COX-2 mRNA \(^27\) and iso-orientin which had selective COX-2 inhibitory activity \(^28\).

### 3.4. In vitro neuroprotective activity

Extracts at concentrations of 25, 50, and 100 µg/mL didn’t result in significant cytotoxicity to neuro-2A cells while concentration of 200 µg/mL showed severe cytotoxicity (Figure 1). So, the neuroprotection against \(H_2O_2\) and \(A_β_{42}\) in neuro-2A cells using the non-cytotoxic concentrations 25, 50, and 100 µg/mL.

![Figure 1](image_url)  
**Figure 1.** Effect of different extracts of \(W.\ filifera\) on cell viability of neuro-2A cells. Values were represented as means ± standard deviations (SD), \(n = 5\). * Significant difference from cell viability of DMSO as a negative control at \(p < 0.01\).

#### 3.5. Effect of the extracts on cell viability of neuro-2A cells after treatment with \(H_2O_2\) as a neurotoxic agent

Treatment of neuro-2A cells with \(H_2O_2\) induced dose-dependent cell cytotoxicity. Doses of 100, 150, and 200 µM of \(H_2O_2\) caused loss of 19.66, 53.77, and 79.78 % of viable neuro-2A cells, respectively. To investigate the neuroprotection of ethanol extracts of different parts of \(W.\ filifera\), \(H_2O_2\) at a dose of 150 µM was used to induce neurotoxicity as it caused about 50 % reduction of cell viability (Figure 2A). The other 2 doses 100 and 200 µM lead to either very low or severe cytotoxicity to the cells, respectively. Catechin as a positive control could significantly (\(p < 0.01\)) increase the cell viability of \(H_2O_2\)-treated cells only at high doses of 50 and 100 µg/mL by 19.3 and 35.2%, respectively (Figure 2B).

Lower concentrations of catechin couldn’t show any significant increase in cell viability. W.F and W.L showed the most potent neuroprotective activities in this assay. W.F manifested significant (\(p < 0.01\)) dose-dependent neuroprotection at 12.5, 25, 50, and 100 µg/mL and increased cell viability by 16.22, 20.80, 26.64 and 28.83%, respectively. Similarly, W.L showed a significant increase in cell viability by 9.57, 13.22, 20.58, and 28.81% at 12.5, 25, 50 and 100 µg/mL, respectively.

The other two extracts; W.B and W.R showed less potent activity at high concentrations; 50 and 100 µg/mL of W.B which increased cell viability by 9.47 and 12.84%,
respectively, and at 100 µg/mL of W.R that increased cell viability by 9.99%. The results of cell viability of neuro-2A cells after treatment with H₂O₂ assay manifested good matching with the DPPH assay of the ethanolic extracts of different parts of W. filifera and could be related to the antioxidant activity together with the phenolic content.

The activity could be explained by the presence of flavonoids such as luteolin which was reported to have an in vivo neuroprotective effect against acetamiprid-induced neurotoxicity in the rat cerebral cortex through increasing the level of antioxidant enzymes such as glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase, decreasing the level of inflammatory mediators such as interleukin-1β, tumor necrosis factor, and nuclear factor kappa B and lowering the oxidants such as nitric oxide and malondialdehyde.

Also, vitexin formerly displayed a neuroprotective effect in rat pups with ischemic injury through inhibition of hypoxia-inducible factor and vascular endothelial growth factor. Furthermore, orientin effectively inhibited H₂O₂-induced accumulation of ROS in rat pheochromocytoma (PC12) cells via inhibition of cell apoptosis and decreasing H₂O₂-induced phosphorylation of mitogen-activated protein kinases and protein kinase B signaling proteins.

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3.6. Effect of the extracts on cell viability of neuro-2A cells after treatment with Aβ₁₋₄₂ as a neurotoxic agent

Twenty-four hours of incubation of neuro-2A cells with different concentrations of Aβ₁₋₄₂ resulted in neurotoxicity and reduction of cell viability in a dose-dependent manner (Figure 3A). The 25 µM concentration of Aβ₁₋₄₂ resulted in a 51.1% decline in cell viability. Epigallocatechin-3-gallate as a positive control could effectively counteract the Aβ₁₋₄₂-induced neurotoxicity by increasing the cell viability of Aβ₁₋₄₂-treated cells (TC) by 10.35, 16.17, 21.89, and 34.99%, at 12.5, 25, 50, and 100 µg/mL, respectively. The most efficient extracts exhibiting neuroprotection against Aβ₁₋₄₂-induced neurotoxicity were W.F followed by W.L that showed more neuroprotection than epicatechin-3-gallate at 12.5, 25, 50, and 100 µg/mL concentrations. The significant neuroprotection (P< 0.01) of W.S and W.B was displayed at higher concentrations; 50 and 100 µg/mL (Figure 3B).

Previous investigation of the activity of the methanol extract of W. filifera seeds (MES) on islet amyloid polypeptide (IAPP) revealed that MES had dose-dependent inhibition of the in vitro IAPP toxic aggregates using Thioflavin T fluorescence assay. Molecular docking study of the isolated compounds illustrated the high binding affinity of the phenolic compounds; procyanidin dimer, P-hydroxy benzoic acid, protocatechuic acid, and catechin to IAPP. To date, other parts of W. filifera were first studied for neuroprotection against Aβ₁₋₄₂ in the present study. The
activity might be related to the presence of vitexin that was reported to have a neuroprotective effect in neuro-2A cells against toxicity induced by Aβ25-35 through enhancing the antioxidant pathway of ROS-mediated apoptosis. Additionally, other previously isolated flavonoids such as; tricin were proved to have inhibitory activity against β-

[Figure 3. (A) Dose-dependent cytotoxic effects of Aβ1-42 in neuro-2A cells. (B) Neuroprotection against Aβ1-42-induced neurotoxicity in neuro-2A cells. Epigallocatechin-3-gallate (E3G) was used as a positive control. Values were represented as means ± standard deviations (SD), n = 5. UT: is the cells treated with DMSO. TC: is the cells treated with Aβ1-42 (25 µM). ** Significant difference from UT in (A) and from cell viability of TC (25 µM) treatment in (B) at * at p < 0.05 and ** p < 0.01.]

4. CONCLUSION

This study showed that the ornamental palm wastes could be exploited for biological investigations as well as solving a part of the problems of agro-wastes. Neuroprotective activities of the ethanolic extracts of W. filifera botanical parts were evaluated. The study indicated that fruitless bunch extract (W.F) exhibited the highest neuroprotective activity as it could potentially inhibit COX-2 and protect against H2O2 and Aβ-induced neurotoxicity in a dose-dependent manner. Although the phenolic content and the antioxidant activity of W.L. were higher than that of W.F., W.F. showed more potent anti-inflammatory activity through inhibition of COX-2 enzyme and neuroprotection by increasing cell viability of H2O2 and Aβ1-42-treated neuro-2A cells. This could be partly explained that the biological activity of natural products is a complex mechanism which may include binding to certain target proteins or inhibition or activation of specific pathways. So, it was supposed that W.F. has other molecular mechanisms for anti-inflammatory and neuroprotective activities in addition to its antioxidant activity, however, the other underlying mechanisms need further investigation. These findings could contribute to the future bio-guided isolation and identification of the neuroprotective compounds of the active extracts in this study (W.F and W.L) as well as studying their neuroprotective mechanisms.

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CONFLICT OF INTEREST

The authors declare that there is no competing interest associated with this work

AUTHOR CONTRIBUTION

Conceptualization, S.E, and A.M; methodology, A.S and A.M; investigations, A.M, A.S, S.E resources, A.M and A.S; writing—original draft preparation, A.M and A.S.; writing—review and editing S.E, A.M, A.S, and E.M. All authors have read and agreed to the published this version of the manuscript.

5. REFERENCES


