Evaluation of the Anti-inflammatory and Antioxidant Activities of Selected Resin Exudates

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ABSTRACT

Plant resins are reported to have high medicinal values due to their content of bioactive metabolites. Few reports were found in the last two decades concerning the chemistry and anti-inflammatory activity of the resins belonging to Eucalyptus and Araucaria genera. Therefore, the exudate resins of Eucalyptus maculata, Araucaria excelsa and Araucaria bidwillii were evaluated for their phenolic and flavonoid content, together with their possible anti-inflammatory potential via carrageenan-induced paw edema in rats at the doses of 100, 200, and 400 mg/kg. The methanol extract of E. maculata (MEME) kino resin was the richest in phenolics (572.82 ± 1.75 µg GAE/mg extract), and flavonoids (11.60 ± 0.01 µg QE/mg extract). The MEME (400 mg/kg) showed the most significant percentage inhibition of paw oedema (50.91%), compared to indomethacin (66.11%). The extract also decreased serum malondialdehyde (MDA) (42%), nuclear factor kappa B (NF-κB) (42%), tumor necrosis factor-alpha (TNF-α) (40.5%), cyclooxygenase-2 (COX-2) (42%) and nitric oxide (NO) (23%). In addition, it increased antioxidant enzymes; reduced glutathione (GSH) (2.6-fold) and superoxide dismutase (SOD) (1.3-fold), compared to control group. Moreover, it displayed a profound antioxidant property with IC50 of 6.83 ± 0.77µg/mL, compared to trolox (IC50 21.18 ± 0.59 µg/mL) by DPPH assay. The methanol extract of E. maculata resin was standardized by HPLC to contain 68.21 mg of 7-O-methyl aromadendrin (MA)/g extract. These findings suggest that E. maculata kino resin is a potent antioxidant with potential therapeutic efficacy for treating inflammation.

Keywords: Eucalyptus maculata, Araucaria excelsa, Araucaria bidwillii, Resin exudate, Inflammatory biomarkers, Antioxidant.

Introduction

Inflammation is a response, triggered by damage to living tissues, to protect them from infection and injury as well as the consequence of such injury i.e. the necrotic cells and tissues. Inflammation is managed mainly with nonsteroidal anti-inflammatory drugs (NSAIDs). Although, these anti-inflammatory drugs can treat many inflammatory conditions, they have numerous side effects such as gastrointestinal injury, hepatotoxicity, sodium retention, obesity and osteoporosis, that might cause serious health problems. As alternatives to synthetic medicines, natural products show good promise, few side effects and proved to be potential candidates to cure inflammatory conditions.

The adverse effects of oxidative stress on human health have become a serious issue. A lack of antioxidants in human diet, which can scavenge the reactive free radicals, facilitates the development of degenerative diseases, including cardiovascular diseases, cancers, neurodegenerative diseases and inflammatory diseases. One solution to this problem is to supplement the diet with antioxidant compounds that are contained in natural plant sources.

Sticky plant secretions that harden when exposed to air are called resins. Resin is composed of mixture of either terpenoids or phenolic compounds, which may be produced in internal ducts or in specialized glands of plants. The genus Araucaria Hook, Family Araucariaceae includes 19 species distributed in the Southern Hemisphere, from Australia and Pacific islands to South America. The resin of this genus was reported to possess gastroprotective, cytotoxic, antimicrobial, anti-pyretic and anti-inflammatory activities. Labdane diterpenes were previously isolated from the resin exudates of A. excelsa and A. bidwillii.

Eucalyptus is one of the most important genera in Family Myrtaceae which includes 132 genera and 5950 species. E. maculata Hook is a synonym of Corrymbia maculata (Hook) K.D.Hill & L.A.S. Johnson indigenous to Australia and cultivated in Egypt. E. maculata resin is rich in bioactive secondary metabolites including phenolics and flavonoids. Although, the importance of E. maculata resin as a rich source of compounds, possessing antioxidant and hepatoprotective activities, the reports of investigation of its phytochemical and pharmacological activities are very rare.

Owing to the potential medicinal value of plant exudates, we selected the resin of three plants for our current study; the oleo resin of A. excelsa (Salis.), A. bidwillii Hook, and the kino resin of E. maculata...
which are abundant in Egypt to investigate their antioxidant and anti-inflammatory activities, and elucidate their possible mechanism(s) of action.

Materials and Methods

Plant material
The kino resin of *E. maculata* and oleo-resin of *A. bidwillii* were collected from the stem of the plants cultivated in the Zoo garden, Giza, Egypt, while oleo-resin of *A. excelsa* was collected from El-Muntaza Palace Garden, Alexandria, Egypt. All plant resins were collected in April 2019 and were identified by Dr. M. Gibali, the Senior Taxonomist in the Department of Botany, National Research Centre, Giza, Egypt and Agriculture Engineer Mrs. Theres Labib, Senior Botanist, Orman botanic garden, Giza, Egypt. Voucher specimens of *E. maculata* (Sp. # EM 2.7.2019), *A. bidwillii* (Sp. # AB 2.7.2019) and *A. excelsa* (Sp. # AE 2.7.2019) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt.

Chemicals and Reagents
All solvents used in the study were of analytical grade and purchased from El-Gomhouria Co. (Cairo, Egypt). Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) reagent, gallic acid, and quercetin were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate, aluminum chloride and potassium acetate were purchased from El-Nasr Company for Pharmaceutical Chemicals, Egypt. Indomethacin capsules (Kahira Pharmaceutical and Chemical Company, Cairo, Egypt), carrageenan (Sigma-Aldrich Chemical Company, USA) and all other chemicals were of high analytical grade and were purchased from local supplier.

Preparation of the extracts
The air-dried powdered resin (10 g each) of *E. maculata* exudate (EME), *A. excelsa* exudate (AEE) and *A. bidwillii* exudate (ABE) were extracted with methanol (3 x 100 mL) using a sonicator for 15 min. Each extract was concentrated under reduced pressure to a constant weight to yield dry methanol residue; (EME, 9.5 g), (AEE, 9.4 g) and (ABE, 8.9 g), respectively. The three extracts were kept in a desiccator over anhydrous CaCl₂.

Chemical analysis
The three methanol extracts were analyzed for their total phenolic content (TPC), total flavonoid content (TFC) and radical scavenging activity using DPPH assay. The most promising extract (*E. maculata*) was standardized using HPLC.

Determination of the total phenolic content
Total phenolics were determined employing Folin Ciocalteu method. Briefly, the total methanol extract of resin (120 g) was partitioned with CH₂Cl₂. The CH₂Cl₂ fraction (7 g) was chromatographed on a VLC column using CH₂Cl₂ / CH₃OH / H₂O (6:1:1) as eluent. The 1:1 fraction (7 g) was chromatographed on a VLC column using CH₂Cl₂ / CH₃OH / H₂O (80:20:10) as eluent. Absorbance was measured at 765 nm. The absorbance was done in triplicate, the mean values were calculated and the results expressed as gallic acid equivalents (GAE) mg extract per kg of sample.

**Equation:**

\[ Y = 0.0041X + 0.0057 \quad R^2 = 0.9996 \]

Where, Y = absorbance, X = concentration (µg/mL) and R² = linear regression coefficient.

**Determination of the total flavonoid content**
Total flavonoid content was measured by the aluminum chloride colorimetric assay described by Sabo et al. Briefly, an aliquot (1 mL) of extract (1 mg/mL) or standard solution of quercetin (5 - 100 µg/mL) was added to 0.1 mL aluminum chloride (10%), 0.1 mL potassium acetate (1 M) solution and 2.8 mL distilled water were added and mixed well, the absorbance was read after 30 min at 415 nm. A blank was prepared in similar way using methanol instead of the sample. The assay was done in triplicate, and the mean values were calculated. Total Flavonoid content in the extract was expressed as quercetin equivalent (µg of quercetin/mg of sample) from the pre-established calibration curve and calculated according to the following equation:

\[ Y = 0.0134X + 0.0165 \quad R^2 = 0.9997 \]

Where, Y = absorbance, X = corresponding concentration (µg/mL) and R² = linear regression coefficient.

**Determination of the radical scavenging activity using DPPH assay**
In vitro antioxidant activity of the investigated extracts were determined using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging assay as previously described. This test provides information on the ability of a compound to donate a hydrogen atom, the number of electrons a given molecule can donate, and on the mechanism of antioxidant action. DPPH solution (0.2 mM) was prepared and 1.0 mL of the solution was added to 3.0 mL of methanol extract at different concentrations (2.5 - 25 µg/mL) of methanol extract. Thirty minutes later, the absorbance was measured at 517 nm. A blank was prepared without adding extract. Trolfox (2.5 - 25 µg/mL) was used as standard. Decreasing absorbance of the reaction mixture indicates higher free radical scavenging activity. The IC₅₀ value of the radical scavenging activity is the effective concentration at which antioxidant activity is 50%. The IC₅₀ of each methanol extract was expressed in µg/mL.

**HPLC analysis of the methanol extract of *E. maculata***

**Sample preparation**
The methanol extract of the resin was subjected to fractionation and column chromatography following previously reported method. Brieﬂy, the total methanol extract of resin (120 g) was partitioned with CH₂Cl₂. The CH₂Cl₂ fraction (7 g) was chromatographed on a VLC column of Si gel 60 (270-400 mesh). Gradient elution was carried out starting with n-hexane, n-hexane/CH₂Cl₂, CH₂Cl₂, and finally CH₂Cl₂/MeOH mixture, up to 100% MeOH. Fraction (1.2 g) eluted with CH₂Cl₂/MeOH (4:1) was further purified on another Si gel column using CH₂Cl₂/MeOH (9:1) to give 300 mg of white microcrystalline powder (compound MA). The chemical structure of the compound was identified by comparing its ¹H and 13C-NMR spectra with those reported in literature.

**Chromatographic conditions**
Standardization of the methanol extract of *E. maculata* was achieved according to a previously reported method with some modification using Agilent 1200 series (USA) HPLC equipped with quaternary pump, auto sampling injector, solvent degasser and UV detector. Chromatographic separation was achieved on Zorbax ODS RP-18 column (250 x 46 mm, 5µm). The mobile phase consisted of acetonitrile (A) and (0.3%) phosphoric acid in water (B) as follows: at 0 min 83% A; at 10 min 65% A; at 15 min 45%; at 17-20 min 100% A. The flow rate was 1.0 mL/min and UV detector at 280 nm. 7-O-Methyl aromadendrin (MA) was used as an external standard. A standard calibration curve of MA showed linearity within dilutions ranging from 10-100 µg/mL. The above-mentioned conditions were used at 16.435 min.

**Biological evaluation**
The anti-inflammatory activity of the methanol extracts of resin exudate of *E. maculata*, *A. excelsa* and *A. bidwillii* were evaluated using carrageenan-induced foot paw oedema model following method of Winter et al. with slight modification.

**Animals**
Sixty-six (11 x 6 in each group) Sprague-Dawley adult male rats, with average age 6-8 weeks and weighing 180 ± 20 g, were purchased from “Egyptian Organization for Biological Products and Vaccines”, Giza, Egypt. All procedures were approved by the “Research Ethics
Committee” for experimental and clinical studies at Faculty of Pharmacy, Cairo University, Egypt” under a serial number MP (2186).

Experimental protocol
Rats were divided into 11 groups (n = 6) of which nine groups were administered the methanol extract of the three plants at three doses (100, 200 and 400 mg/kg, p.o) and the remaining two groups of animals received carrageenan (0.1 mL of 1%, freshly prepared) and indomethacin as standard drug (5 mg/kg, i.p). One hour after drug administration, inflammation was induced by sub planter injection of 0.1 mL of carrageenan (1% freshly prepared suspension) in saline into the paws of right hind limb of the rats of all groups.

Determination of the percentage oedema and percentage inhibition of oedema (1%) The percentage oedema at the end of the experiment and percentage inhibition of the oedema (%) of each group were calculated as follows:

\[ \text{Oedema} = \frac{PT_1 - PT_2}{PT_1} \times 100 \]

\[ \text{Inhibition} (\%) = (1 - \frac{E_d}{E_c}) \times 100 \]

Where, \( PT_1 \) and \( PT_2 \) are the paw diameter (mm) before and after carrageenan injection, respectively, \( E_d \) and \( E_c \) are the mean percentage oedema of the treated and carrageenan group, respectively.

Blood sampling
Four hours after carrageenan injection, animals which received E. maculata resin extract at dose of 400 mg/kg (which showed the highest activity) were anesthetized using thiopental, and then blood samples were collected from the retro-orbital venous plexus. The serum samples were obtained by centrifugation (1000 x g for 15 min) and the serum was stored at -80°C until analysis.

Estimation of oxidative stress markers.
Malondialdehyde (MDA) is an index of lipid peroxidation, its content was quantified by reaction with thiobarbituric acid (TBA) and absorbance was measured spectrophotometrically at 532 nm according to the method reported previously. Reduced glutathione (GSH) content was determined according to the method reported previously. The absorbance of the product was measured spectrophotometrically at 412 nm. Superoxide dismutase (SOD) activity was determined by observing the SOD-inhibitable auto-oxidation of pyrogallol, as described previously.

Determination of inflammatory biomarkers
Tumour necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2) levels were assayed in serum using rat sandwich ELISA kits (Cusabio Biotech Co, USA). Additionally, Nuclear factor kappa B (NFκB-B) level was measured by sandwich ELISA kits (MyBiosource San Diego, CA, USA) according to the manufacturer’s instructions.

Determination of total nitrite/nitrate (NOx) markers
The total nitrite/nitrate (NOx) products, an indicator of NO synthesis, were measured according to the methods described previously.

Statistical Analysis
Data were expressed as mean ± standard deviation (SD), where n= no. of rats. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons post hoc test. The level of significance was set at p < 0.05. The GraphPad Prism V 6 (GraphPad Software Inc., San Diego, CA, USA) was used.

Results and Discussion

Phenolic and flavonoid contents
The MEME kino resin showed the highest phenolics (572.82 ± 1.75 µg GAE/mg extract) and flavonoid content (11.60 ± 0.01 µg quercetin/mg extract) among the tested resins. The phenolic content of the methanol extracts of A. excelsa (AEE) and A. bidwillii (ABE) resins was 30.04 ± 0.32 and 12.35 ± 0.01 µg GAE/mg extract, respectively, while their flavonoid content was 4.09 ± 0.01 and 6.55 ± 0.31 µg quercetin/mg extract, respectively.

DPPH scavenging activity
The MEME resin reduced the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine with IC50 value of 6.83 ± 0.77 µg/mL and was found to be more potent than the positive control; trolox (IC50 value of 21.18 ± 0.59). However, the methanol extracts of resin of AEE and ABE showed IC50 values of 617.86 ± 0.91 and 3796 ± 0.84 µg/mL.

Standardization of the most potent extract
Standardization of the MEME kino resin was made using HPLC utilizing 7-O-methyl aromadendrin (MA) as reference standard. The concentration of compound MA was determined from standard calibration curve to be 68.21 mg/g MEME.

Biological evaluation
Anti-inflammatory activity (carrageenan-induced paw oedema)
After 4 h of carrageenan administration, all treated groups that received methanol extract of the three plants at three doses (100, 200 and 400 mg/kg) significantly decreased the paw oedema at P < 0.05 and percentage oedema (Figure 2), when compared to the control group which received only carrageenan. The magnitude of the decrease was higher for the highest dose (400 mg/kg) among all tested groups, suggesting a dose-dependent effect. Interestingly, the MEME at 400 mg/kg showed E% of 35.5 ± 1.07, and a higher value of percentage inhibition of paw oedema (1%) of 50.91, while indomethacin group showed 66.1% (Figure 3A-B). These results clearly showed that the methanol extract of E. maculata kino resin (400 mg/kg) had the highest anti-inflammatory activity.

Effect of MEME resin on antioxidant status
The role of E. maculata in enhancing the antioxidant status was investigated by measuring the enzyme levels of MDA (Figure 4A), GSH (Figure 4B) and SOD (Figure 4C) in serum. Indomethacin administration attenuated the oxidative stress induced by carrageenan through decreasing MDA level by 47.5% from the control group (carrageenan only) and increasing the antioxidant enzyme GSH by 3.9-fold and SOD by 1.5-fold compared to the control group. The MEME resin (400 mg/kg) administration significantly attenuated the oxidative stress induced by carrageenan through decreasing MDA level by 42% from carrageenan group and increasing both antioxidant enzyme GSH and SOD levels by 2.6-, and 1.3-fold, respectively of carrageenan group. Moreover, MEME (400 mg/kg) showed no significant difference in the level of MDA and SOD when compared with indomethacin group.

Figure 1: Chemical structure of compound MA (7-O-Methyl aromadendrin)
Effect of MEME resin on TNF-α, NFκB, COX-2 and NO serum levels

To investigate the role of MEME resin as an anti-inflammatory, the NFκB- and TNF-α- (Figure 5A), COX-2 (Figure 5B) and NO (Figure 5D), the key regulators in inflammation were measured in serum. Treatment with indomethacin significantly reduced the serum levels of inflammatory biomarkers by 60%, 59%, 65% and 43%, respectively, from carrageenan group. Treatment with the methanol extract of MEME resin (400 mg/kg) significantly reduced the serum levels of inflammatory biomarkers NFκB, TNF-α, COX-2, and NO by 42.7, 40.5, 42 and 23%, respectively compared to the control group.

Carrageenan-induced severe oedema in rats includes two different phases: the initial phase (0-1 h) of oedema is attributed to the release of histamine, bradykinin and serotonin which mediates the increased synthesis of prostaglandins from surrounding tissues of the injured area. The second phase (3-5 h) is mainly mediated by prostaglandin, cytokines, cyclooxygenase, nitric oxide, and neutrophil derived free radicals.

With the aim of finding new natural remedies for the management of inflammation, three exudate resin extracts were screened for their potential anti-inflammatory activity using carrageenan-induced rat paw oedema model. The results showed that oral administration of methanol extracts of E. maculata kinos, A. bidwillii and A. excelsa oleo-resins (100, 200 and 400 mg/kg) pretreatment produced a significant improvement in the paw inflammation. The MEME at a dose of 400 mg/kg demonstrated the most potent oedema inhibition (50.9%) compared to control group. In addition, EME resin possessed higher antioxidative potential evaluated by DPH assay, which can be attributed to its higher phenolic and flavonoid contents than the other tested resins. To get a deeper insight about the mechanism involved in alleviating inflammation, attenuation in oxidant status and inflammatory biomarkers in serum were further studied. Cyclooxygenase-2 (COX-2) represents a vital key enzyme in inflammation and is considered as a rate-limiting enzyme, which catalyzes prostaglandins production, which is responsible for the formation of inflammatory mediators. NO is also responsible for vasodilatation, the increase in vascular permeability and oedema formation at the site of inflammation. NFκB is recognized to be the main transcription factor regulating the expression of proinflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α and interleukin-1 (IL-1). TNF-α, a mediator of carrageenan-induced inflammation, is able to induce the further release of kinins and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting inflammatory response. Hence, inhibition of these markers leads to reduction of inflammatory conditions. Pretreatment with MEME resin significantly decreased these markers in serum after carrageenan injection. In a number of pathophysiological conditions associated with inflammation or oxidative stress, reactive oxygen species (ROS) have been reported to mediate cell damage via a number of mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of antioxidant enzymes such as GSH and SOD. MDA production after carrageenan injection is due to free radical attacking plasma membrane. Thus, inflammation would result in the accumulation of MDA and reduction of anti-oxidative status by reduction of GSH and SOD activity. Additionally, the level of MDA was decreased significantly, although the level of GSH and SOD enzyme were significantly increased from control group by treatment with the MEME resin (400 mg/kg), confirming its anti-oxidative effect. These results are greatly supported by the previously reported antioxidant activity of MEME kinos resin. The potent anti-inflammatory and antioxidant activities of the MEME kinos resin might be attributed to its high phenolic and flavonoid contents. Therefore, it may be used as a potential agent in the management of diseases in which free radical formation is a pathogenic factor.

Figure 2: Percentage of oedema at 4h in carrageenan-induced oedema for the extracts of three resin exudates. EME: methanol extract of Eucalyptus maculata resin; ABE: methanol extract of Araucaria bidwillii oleo-resin; AEE: methanol extract of Araucaria excelsa resin.

Data are expressed as mean ± SD, n = 6, P < 0.05 (* compared to carrageenan group, # compared to indomethacin group) using regular one-way ANOVA followed by Tukey’s multiple comparisons test.

Figure 3: Effect of E. maculata resin extract on paw thickness and % of oedema at 4h in carrageenan-induced oedema. A: Paw thickness at 4h (mm); B: % of oedema at 4h Data is expressed as mean ± SD, n = 6, P < 0.05 (* compared to carrageenan group, # compared to indomethacin group) using regular one-way ANOVA followed by Tukey’s multiple comparisons test.
Figure 4: Effect of *E.* maculata resin extract (400 mg/kg) at 4h on anti-oxidant parameters. A: Malondialdehyde (MDA); B: Reduced glutathione (GSH); C: Superoxide dismutase (SOD).

Data is expressed as mean ± SD, n= 6, *P < 0.05 (* compared to carrageenan group, # compared to indomethacin group) using regular one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 5: Effect of *E.* maculata resin extract (400 mg/kg) at 4h on inflammatory markers. A: (NF-κB); B: (TNF-α); C: (COX-2); D: (NO).

Data is expressed as mean ± SD, n= 6, *P < 0.05 (* compared to carrageenan group, # compared to indomethacin group) using regular one-way ANOVA followed by Tukey's multiple comparisons test.
Figure 6: HPLC chromatogram of the methanol extract of E. maculata resin. (MA): 7-O-Methyl aromadendrin

Conclusion
In the current study, we evaluated the antioxidant and anti-inflammatory activities of E. maculata kino resin, A. bidwillii and A. excelsa resin exudates. Methanol extract of E. maculata (400 mg/kg) showed the highest antioxidant activity. The results confirmed that the methanol extract of E. maculata kino resin, A. bidwillii and A. excelsa oleo-resin (100, 200 and 400 mg/kg) reduced carrageenan-induced paw oedema in rats. The methanol extract of E. maculata kino resin (400 mg/kg) was the most potent through its anti-inflammatory and antioxidant activity in a TNF-α, NFκB and COX-2 dependent manner and this can be attributed to its high content in phenolics.

Conflict of interest
The authors declare no conflict of interest

Authors’ Declaration
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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