In vitro antioxidant and DNA damage inhibition activity of ethanolic extract of Araucaria heterophylla (Salisb.) Franco (Norfolk Island pine)

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Abstract

Cancer is now one of the leading causes of death worldwide. One of the main causes, however, is attributed to DNA damage brought about by mutations. These mutations possibly occur due to destructive chain reactions stimulated by increased free radicals that could have been caused by exposure to external factors. Thus, there had been various studies to help prevent its continuous emergence, such as the use of plants as sources of bioactive metabolites. This study used the leaf extract of *Araucaria heterophylla (Salisb.) Franco*, commonly known as Norfolk Island Pine. Phytochemical screening of this plant's leaf extract showed the presence of flavonoids and exhibited significant activities in DPPH radical scavenging, total antioxidant, H_2O_2 scavenging and reducing power assays. The 75 ug/mL n-hexane extract showed the highest DPPH scavenging percentage of 97%. As for the total antioxidant activity, the 1000 ug/mL ethanol extract had the highest activity. More so, for hydrogen peroxide scavenging, the 50 ug/mL n-hexane extract had the highest hydrogen peroxide scavenging of 95.46%. Meanwhile, for reducing power, the 1000 ug/mL n-hexane extract demonstrated the highest reducing power activity. Lastly, the n-hexane extract demonstrated DNA damage inhibition potential. With these results and findings, this plant could possibly be of use in cancer prevention medicine in the future.

Keywords: *antioxidants, cancer, oxidative stress, Araucaria heterophylla (Salisb.) Franco, flavonoids*

INTRODUCTION

Antioxidants endow an important role in protecting DNA from various reactive oxygen species mediated damages (George, et al., 2014). These molecules stop free radical reactions and prolong or obstruct cellular damage (Nimse & Pal, 2015). DNA damages are then due to the free radicals. Being unstable, these free radicals have the tendency to get electrons from their neighboring molecules and later on stimulate a destructive chain reaction that can eventually cause oxidative stress in the body (Odchimar, et al., 2016). This oxidative stress, however, was linked as one of the causes of various diseases like cancer (Rahal et al., 2014).

Cancer is a group of disease with an abnormal growth and spread of cells. The reason for cancer is still unknown but there are external factors that contribute to its development. These include lifestyle factors, such as smoking and obesity, and non-modifiable factors, such as inherited genetic mutations, hormones, and immune conditions. (American Cancer Society, 2017). Oxidative damage, being one of the causes of cancer, is then due to the changes in homeostasis. This leads to increased production of free radicals which eventually surpasses the detoxifying ability of the local tissues, hence enabling these excess free radicals to cause damage. Further generation of free radicals can then be elicited by external factors such as smoking, pollution and sunlight which also contribute to the development of cancer (Rahal et al., 2014).

Plants are then said to be potential sources of antioxidants that are generally safer than the commercially-made antioxidants. In fact, medicinal plants are excellent sources of phytochemicals that are able to reduce the oxidative damage to cells that causes diseases (Nasri & Rafieian-Kopaei, 2014). *Araucaria heterophylla (Salisb.) Franco* which is known as Norfolk Island pine was used in this study. In addition to ornamental and timber purposes, some *Araucaria* species have medicinal purposes as they have antidepressant, anti-viral, anti-inflammatory, neuroprotective, antipyretic, anticoagulant, and antimicrobial activities (Verma et al., 2014). More so, cytotoxic and gastroprotective activities are present in the resins of this genus. Likewise, diterpenes as well as lignans had been isolated from the resin exudates of various *Araucaria* species including this exact specie (Abdel-Sattar, et al., 2009). Thus, the extract of *Araucaria heterophylla (Salisb.) Franco* was evaluated in this study for antioxidant capacity as well as DNA damage inhibition effect and underwent phytochemical screening, DPPH

scavenging, total antioxidant activity, hydrogen peroxide scavenging, reducing power and DNA damage inhibition test.

This study may help in the medical field in the future by preventing different kinds of disease caused by DNA damaging and mutation.

Figure 1 *Araucaria heterophylla (Salisb.) Franco* (Norfolk Island Pine).

MATERIALS AND METHODS

Plant material

The leaves of *Araucaria heterophylla (Salisb.) Franco* were collected from Tagaytay, Cavite and was authenticated at Bureau of Plant Industry, Malate, Manila (See Appendix A).

Plant collection and extraction

Fresh mature leaves (100 gm) of *Araucaria heterophylla (Salisb.) Franco* collected from Tagaytay, Cavite were washed with distilled water to remove soil particles. The leaves were then placed in the oven and dried at 35°C for 48 hours (Jusril, et al., 2016). Next, the dried leaves were pulverized with the use of a grinder. The leaf powder (10 gm) was then soaked in ethanol at room temperature for 24 hours. After that, it was loaded on a shaker at a speed of 120 rpm for another 24 hours. The mixtures were then filtered by using Whatman number 1 filter paper. The filtrate was next concentrated under reduced pressure with a rotary evaporator and was later exposed under the laminar flow hood. Lastly, the dried extract was placed in an air tight receptacle and stored at room temperature for further use (Verma, et al., 2014).

As for bioassays, another 100 g of the dried leaves were powdered and extracted with n-hexane and methanol (200 mL/solvent) in a Soxhlet extractor for 2 hours. The extracts were then concentrated until dryness under reduced pressure to produce two oily greenish brown extracts. After that, those were exposed under the laminar flow hood and were then kept as well as refrigerated for further use (Michael, et al., 2010).

Phytochemical screening

Detection of flavonoids

Alkaline reagent test: A 0.5 g ethanol extract was dissolved in 100 mL distilled water while placed in a water bath. Few drops of sodium hydroxide solution were used to treat the extract. Appearance of intense yellow color which turned colorless after dilute acid was added is an indication of the presence of flavonoids (Tiwari, et al., 2011).

Detection of diterpenes

Copper acetate test: A 0.5 g ethanol extract was dissolved in 100 mL distilled water while placed in a water bath. Then, 3-4 drops of acetate solution were added to the extract and appearance of an emerald green color is an indication of the presence of diterpenes (Tiwari, et al., 2011).

DPPH scavenging activity

In this phase, the extracts were diluted in ethanol, methanol and n-hexane to 25, 50, 75, and 100 µg/mL, then 2 mL of each dilution was mixed with 1 mL of DPPH solution (0.2 mM/mL in respective extracts) and mixed thoroughly. Next, the mixture were incubated for 20 minutes in the dark at 20˚C, and the absorbance was measured at 517 nm in an ultraviolet-visible (UV-Vis) spectrophotometer with the solvents as the blank. Ascorbic acid mixed with DPPH in increasing concentrations similar with that of the extracts was used as the experimental controls.

Experiments were performed in triplicates at each concentration. Finally, the percentage scavenging from DPPH was calculated. A decreased absorbance will indicate positive for the test.

The DPPH scavenging percentage by the extracts was calculated according to this formula:

$$\text{DPPH radical scavenging\%} = [{}^{(Ac - At)} / {}_{Ac}] \times 100$$

Wherein Ac is the absorbance of the control (DPPH) while At is the absorbance of test sample as well as standards (Verma, Kumar, & Shrivastava, 2014).

Total antioxidant activity

A 1 mL of the extracts (125, 250, 500, and 1000 µg/mL) was combined with 3 mL of the reaction mixture which contained 10 mL of conc. H_2SO_4 and 1.005 g sodium phosphate monobasic as well as 1.47 g ammonium molybdate dissolved in 290 mL of water. The mixture was placed in a water bath at 95˚C for an hour. Absorbance was measured in a UV-Vis spectrophotometer at 695 nm and a solution with 3 mL of the reaction mixture and 1 mL distilled water was used as the blank. The ascorbic acid mixed with the reaction mixture in increasing concentration similar with that of the extracts was then used as the positive control. The experiment was done in triplicates for each concentration. A decreased absorbance will indicate positive for the test (Verma, et al., 2014).

Scavenging of hydrogen peroxide

A 2 mL of each extracts after dilution (25, 50, 75, and 100 µg/mL) were added to 1.2 mL of H_2O_2 solution (40 mM/L in phosphate buffer, pH 7.4) and was incubated for 10 minutes. Using a UV-Vis spectrophotometer, the absorbance of the solution was measured at 230 nm against the blank which was the plant extracts without H_2O_2. Ascorbic acid with H_2O_2 solution in increasing concentration similar with that of the extracts was then utilized as the positive control. The experiment was also done in triplicates at each concentration. A decreased absorbance will indicate positive for the test.

The percentage scavenging of hydrogen peroxide of the extracts was calculated using this equation:

$$\% \text{ Scavenging of } H_2O_2 = [{}^{(Ac - As)}/_{Ac}] \times 100$$

wherein Ac is the absorbance of the control while as is the absorbance in the presence of the sample as well as standards (Verma, Kumar, & Shrivastava, 2014).

Reducing power

All the plant extracts after a series of dilution (125, 250, 500, and 1000 µg/mL) was combined with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL 1% $K_3Fe(CN)_6$ and was incubated at 50˚C for 20 minutes. After addition of 2.5 mL trichloroacetic acid

(10%) to the mixture, it was centrifuged at 4˚C at 3000 rpm for 10 minutes in a cooling centrifuge. Then, 2.5 mL of the supernatant was mixed with an equal volume of distilled water and 0.5 mL ferric chloride (0.1%). In a UV-Vis spectrophotometer, the absorbance was measured at 700 nm with the solvents as the blanks. Ascorbic acid with 2.5 mL of supernatant, 2.5 mL distilled water and 0.5 mL ferric chloride in increasing concentration similar with that of the extracts was utilized as the experimental control. The experiment was also done in triplicates at each concentration. An increased absorbance will indicate positive for the test (Verma, et al., 2014).

DNA damage inhibition

The extract with the highest DPPH scavenging percentage, n-hexane, was used in inhibiting DNA damage of the leaves of *Araucaria heterophylla (Salisb.) Franco* and it was tested by photolysing H_2O_2 with UV radiation in the presence of pBR322 plasmid DNA and performing agarose gel electrophoresis with the irradiated DNA. To 1-µL aliquots of pBR322 plasmid DNA in four microcentrifuge tubes, 50 µL of extract were added to the three while nuclease free water was added to the negative control. All tubes received 4 µL of 3% H_2O_2 and were placed directly on the surface of a UV transilluminator (300 nm) for 10 minutes at room temperature. A 1 uL of loading buffer was next added to all microcentrifuge tubes. All samples were next analyzed by gel electrophoresis on 1% agarose gel (containing Sybr Green) in TAE buffer with a voltage of 120 for 30 minutes (Verma, Kumar, & Shrivastava, 2014).

Statistical analysis

The results for radical scavenging activity, total antioxidant activity, H_2O_2 scavenging activity, reducing power and total phenolic content of the ethanolic extract of *Araucaria heterophylla (Salisb.) Franco* leaves were expressed as mean ± standard deviations of the responses of three replicates per sample. Statistical significance between the groups were determined by one-way ANOVA coupled with Tukey's post hoc test at $p < 0.05$. Statistical analysis was been performed with Microsoft Excel and GraphPad Prism 5 (Verma, et al., 2014).

RESULTS AND DISCUSSION

The produced extracts from the leaves of *Araucaria heterophylla* appeared oily greenish brown which weighed approximately 1.5-2 grams.

Figure 2. Extracts yield of *Araucaria heterophylla (Salisb.) Franco*

Phytochemical screening

Preliminary screening of phytochemicals was performed as this is a crucial step in the detection of bioactive components that can be found in plants which could possibly lead to future discovery and development of drugs (Yadav, et al., 2014). The phytochemical analysis of the ethanolic extract of *Araucaria heterophylla (Salisb.) Franco* confirmed the presence of flavonoids and the absence of diterpenes as shown in Table1. With that, there are increased possibilities of having a high antioxidant activity in the extract as well as anti-cancer activities. This is because flavonoids' protective impacts in the biological systems are attributed to their antioxidant properties. More so, flavonoids have wide spectrum of pharmacological activities like anti-cancer. These meddle in cancer progression by modifying various enzymes as well as receptors in signal transduction pathways that is involved with cellular proliferation (Ravishankar, et al., 2013).

Table 1. Qualitative Phytochemical screening

Flavonoids	**+**
Diterpenes	**-**

DPPH radical scavenging activity

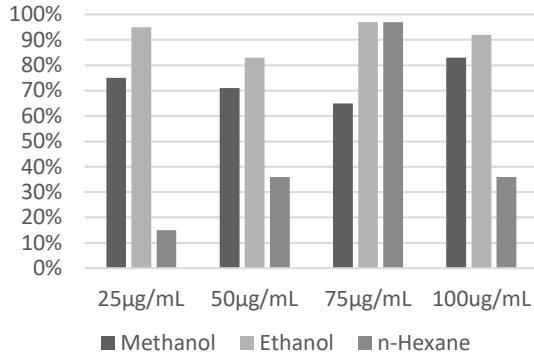

Figure 3. DPPH Scavenging Activity of Differect Extracts at Different Concentrations

Free radicals play a major role in numerous chronic pathologies. A considered model of lipophilic radical is the DPPH (Abbas, et al., 2013). It is defined as a stable free radical that accepts an electron or hydrogen radical in order to be a stable diamagnetic molecule. It has also been significantly used as a free radical for evaluating reducing substances and is a beneficial reagent for investigating the free radical-scavenging activities of compounds (Sathya, et al., 2013). Antioxidants react with DPPH by reducing the number of DPPH free radical to the number of their hydroxyl groups (Abbas, et al., 2013). It showed a purple color with methanol, ethanol and n-hexane, while with a presence of an antioxidant proton donor, the color is reduced to yellow. The results are shown in Figure 2. The scavenging activity of n-hexane and ethanol at 75 ug/mL concentration showed the highest DPPH scavenging percentage. This is also found in agreement with a study in which hexane ($60.52 \pm 0.39\%$) as well as ethanol ($56.78 \pm 1.37\%$) likewise exhibited the highest DPPH scavenging percentage (Ashraf, Rashid, Sarfraz, & Shahid, 2014).

In this study, the various extracts of *A. heterophylla* leaves were evaluated for its DPPH radical scavenging activity in a dose dependent manner. The multiple comparisons on concentration per extract, for methanol and ethanol all, concentrations were significant ($p<0.05$) when compared to n-hexane which indicates that in increasing concentrations there is also an increasing DPPH scavenging activity. Meanwhile, for n-hexane,

concentrations 50 and 75 ug/mL were found to be the same and were therefore non-significant whereas all the other concentrations were significant ($p<0.05$). With that, the plant extract possibly possesses capability of donating hydrogen to a free radical to eliminate odd electron responsible for the reactivity of the radical (Aiyegoro & Okoh, 2010).

Total antioxidant activity

Molybdate (VI) reduction to molybdate (V) in an acidic pH by the extract and green phosphate complex development quantified spectrophotometrically at 695 nm is the basis of total antioxidant test (Verma, et al., 2014). The total antioxidant activity is shown in Figure 3. The 1000 ug/mL concentration of ethanol was found having the highest total antioxidant activity. This is also found in agreement with a study by Do et al. (2014).

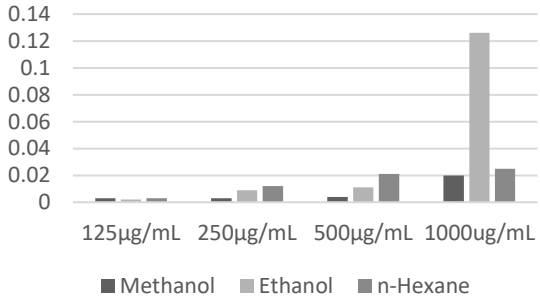

Figure 4. Total Antioxidant activity of Extracts at Different Concentrations

In this study, the various extracts of *A. heterophylla* leaves were evaluated for its total antioxidant activity in a dose dependent manner. Multiple comparisons on concentration per extract were also done for methanol, concentrations of 125 and 250 ug/mL, 125 and 500 ug/mL, as well as 250 and 500 ug/mL were found to be non-significant while all the others were significant ($p<0.05$). As for ethanol and hexane, all concentrations were found to be significant ($p<0.05$) indicating that in increasing concentrations there is likewise an increasing total antioxidant activity. The ethanolic extract demonstrated the highest electron-donating capacity expressing its ability to terminate a chain reaction, producing a more stable non-reactive products from a free radical species (Batool et al., 2010). The results suggested that this may be brought by the phenolic compounds present on the extract (Falleh et al., 2008). The

phosphomolybdenum method is used for evaluation of both water-soluble and fat-soluble antioxidant capability and is for screening samples of very dissimilar origins as well as compositions in search for natural sources of vitamin E and other strong antioxidants (Latayada & Uy, 2016).

Scavenging of hydrogen peroxide

Evaluation of the samples' antioxidant property is done by using the method hydrogen peroxide radical scavenging activity. The ability to scavenge H_2O_2 was determined thru a decrease in absorbance in the test. A decrease in absorbance then shows an increase scavenging ability of the extract. In this assay, n-hexane exhibited the highest % scavenging of H_2O_2 followed by ethanol and methanol showing the least scavenging effect. The H_2O_2 scavenging activity is shown in Figure 4. Results agrees with a study of Anantharaman, et al., (2013) wherein the hydrogen peroxide radical scavenging activity was found to be maximum in hexane extract (55.3%) of *C. marina*.

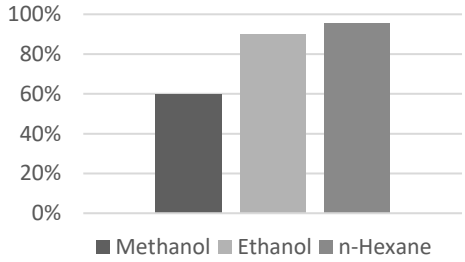

Figure 5. Percentage of Scavenging Activity of Different…

In this study, the various extracts of *A. heterophylla* leaves were evaluated for its hydrogen peroxide radical scavenging activity in a dose dependent manner. Multiple comparisons on concentration per extract were also done for methanol, concentrations of 25 and 50 ug/mL, 25 and 75 ug/mL, as well as 50 and 75 ug/mL were found to be non-significant while all the others were significant. As for ethanol and hexane, all concentrations were found to be significant ($p<0.05$) indicating that in increasing concentrations there is also an increasing hydrogen peroxide scavenging activity. In addition, hydrogen peroxide does not typically react with cells, yet it could give rise to having hydroxyl radicals. With that, compounds capable of electron donation to hydrogen peroxide which could then reduce it to water can block hydroxyl radical production and protect the

cell. This electron donation ability of the sample is then directly related to the antioxidant property (Verma, et al., 2014). Hence, H_2O_2 removal is crucial for antioxidant defense in cell (Keser, et al., 2012).

Reducing power

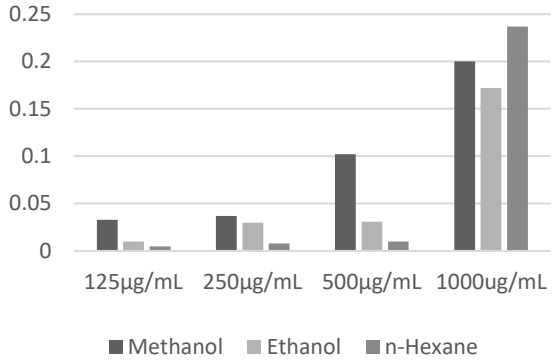

Figure 6. Reducing Power of Different Extarcts

Fe (III) reduction to Fe (II) by electron donation ability of compounds is the basis of reducing power activity. This said reduction of the ions leads to a yellow to bluish green color formation in the reaction mixture which is then measured spectrophotometrically at 700 nm (Verma, Kumar, & Shrivastava, 2014). The reducing power of the extract is shown in figure 5. The 1000 ug/mL concentration of n-hexane was found having the highest reducing power activity. This is also found in agreement with a study (Ashraf, et al., 2014). Reducing capacity of the three investigated extracts increases with increase in concentration. A significant difference among reducing activities of each extract was observed at different concentrations and the reducing power of the hexane extract was significantly higher than methanol and the other extracts.

In this study, the various extracts of *A. heterophylla* leaves were evaluated for its reducing power activity in a dose dependent manner. The multiple comparisons on concentration per extract were also done for methanol, all concentrations were significant which indicates that in increasing concentrations there is likewise an increasing reducing power activity. As for ethanol and n-hexane, both of the respective 50 and 75 ug/mL were found to be insignificant while all the other concentrations were

significant. This increased reducing power activity could be attributed to the compound's reducing capability being a significant indicator of its potential antioxidant activity (Oktay, et al., 2002).

DNA damage inhibition activity

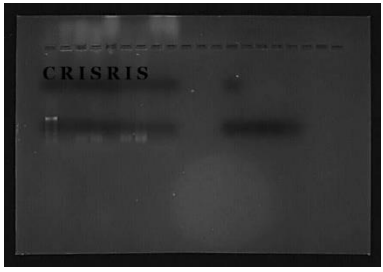

Figure 7. Electrophoretic pattern with DNA gel stain. C, control; R, radiated DNA; I, irradiated DNA; S, extract treated DNA

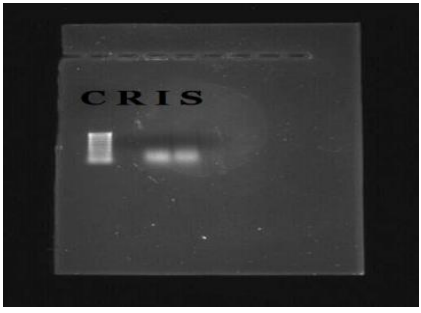

Figure 8. Electrophoretic pattern with DNA gel stain. C, control; R, radiated DNA; I, irradiated DNA; S, extract treated DNA

In this study, n-hexane extract's DNA damage inhibition property was assessed and reported. Figures 8 and 9 displayed the electrophoretic patterns of pBR322 DNA after UV photolysis of H_2O_2 wherein there is absence in the negative control and presence in 2 out of the 3 treated with extracts. With this, it could be concluded that the extract does have a significant protective property against DNA damage although 1 of the treated samples, showed inconsistent results. This error could then be due to pipetting errors or even with insolubilities, for instance incomplete solubility of nuclease free water with the actual extract (Adams, 2013). The result infers that the protection from DNA damage was due to the presence of flavonoids in hexane

extracts of *Araucaria heterophylla*. According to Girish (2016), the C-glycosyl flavones offered maximum protection against radical induced DNA. Recent studies show that dietary flavonoid is efficient in protecting various carcinogen-induced toxicity and has mechanism of DNA damage response and repair process (George, 2017). The results of the study showed potential for DNA damage inhibition of the plant's n-hexane extract.

CONCLUSION

Araucaria heterophylla leaves has potent antioxidant activity attributable to the presence of certain phytochemical compounds needed for protection against oxidative stress. The extract also showed protective DNA damage inhibition ability which is significant in cancer prevention.

RECOMMENDATION

It is highly advised that upon using the same plant extraction using the Soxhlet extractor must be repeated multiple times and it must be of high concentration to attain better results. Also, use different solvents that are miscible with the reagents used in the assays is recommended.

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