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Toxicity and antioxidant properties of the extracts of *Prunus grisea* (C. Muell.) Kalkmleaves

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Abstract

Decoction and various extracts (hexane, chloroform and aqueous) were prepared from the leaves of *P. grisea*. These extracts were subjected to evaluation of toxicity using the brine shrimp lethality test and their antioxidant properties were determined different methods namely the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical- scavenging test, the phosphomolybdenum method for the total antioxidant capacity test and the Folin-Ciocalteu method for the total phenolics test. The chloroform extract (PgC) exhibited the highest toxicity to the brine shrimp *Artemia salina* with LC_{50} value of < 10 ppm. Results also indicated the decoction (PgD) of *P. grisea* leaves to have the most potent antioxidant properties, with percent antiradical activity of 92.85% at 500 ppm, total antioxidant capacity of 110.42 Ascorbic Acid Equivalents (AAE) and 147.14 ButylatedHydroxytolene Equivalents (BHTE) and total phenolics content of 319.00 Gallic Acid Equivalence (GAE). These data may help establish the medicinal potential of this plant and may serve as a guide for further biological and chemical investigations on this plant.

Key words : medicinal plant, radical scavenging, total phenolics, phosphomolybdenum

INTRODUCTION

Over decades, traditional medicine which includes the use of herbal plants has been gaining widespread economic and health system importance[1]. According to the World Health Organization, most of the population of many developing countries regularly use remedies based on plants as their main form of healthcare[2]. The Philippines together with Hong Kong, Malaysia, Mongolia, Singapore, Thailand, and Australia, are classified as a countries "supportive" of traditional medicine according to the World Health Organization. This implies that the government recognizes the role played by traditional medicine, supports its proper use, initiates efforts to bring proven traditional medicine into the formal health service system and takes measures to control its safe practice[3].

Prunus grisea (C. Muell.)Kalkm is identified as native plant variety in the Philippines, Malaysia, Singapore, Taiwan and China[4]. This plant was reported to contain saponins, triterpenes and steroids in a phytochemical survey of 212 plant species in Malaysia[5]. Detailed literature information on this plant is scant but since the Subanen indigenous people of Southern Philippines considered it as having therapeutic properties then, further investigations can be done concentrating on its potentials as a healthcare product. These include determination of its toxicity and antioxidant capabilities in order to provide facts regarding its application as herbal medicine.

The *in vivo* lethality in a simple zoological organism, such as the brine shrimp lethality test can be used as a simple tool to evaluate physiologically active plant extracts, where one of the simplest biological responses to monitor is through lethality[6-7]. This general bioassay detects a broad range of biological activities and a predictive method used for identifying cytotoxicity and pesticidal activity[8-17].

Reactive oxygen species (ROS) are commonly produced as by-products of biological reaction or from exogenous factors, which includes superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide[18-19]. Some of these ROS positively function *in vivo*, such as in energy production, phagocytosis, regulation of cell growth and intercellular signaling or synthesis of biologically important compounds[18]. However, these species can also attack lipids in cell membranes and DNA[20]. These attacks lead to oxidations causing membrane damage such as membrane lipid peroxidation, decrease in membrane fluidity and DNA mutation leading to cancer[21-22]. Potential scavengers of ROS may provide possible preventive intervention for free radical-mediated diseases[22-23]. Recent studies have shown that certain plant products including polyphenolic substances (like the flavonoids and tannins) and various plant or herb extracts exhibit antioxidant actions[24]. Antioxidants possess higher oxidative potential which can protect or act as chain inhibitors of radical inducted decomposition. Antioxidants break up the propagation chains by acting as a hydrogen atom or an electron donor to the free radical and as an acceptor of the excess energy released by the activated molecule[25]. At present, there is a growing interest on finding naturally occurring antioxidants to be utilized in foods and medicinal materials in replacement of synthetic antioxidants which are being regulated because of their reported side effects such as carcinogenicity[26]. Antioxidants derived from natural resources possess versatility in terms of multitude and magnitude of activity[27].

There are many different methods for determining antioxidant function which rely on different generators of free radicals, acting by different mechanisms. Presently, a combination of methods is being suggested in assessing antioxidant activities in vitro in order to cover all the aspects of antioxidant activities[28]. The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay is known as one of the widely used test in determining the scavenging activity of natural compounds due to its low-cost and simplicity[29]. In the DPPH assay, the antioxidants are able to reduce the stable radical DPPH to non-radical form,

DPPH-H. The color changes from purple to yellow after reduction, which can be measured by its decrease of absorbance at wavelength 517 nm. Radical scavenging activity increases with increasing percentage of the free radical inhibition[30-32]. The phosphomolydenum method is known to be employed in determining the total antioxidant capacity of plant extracts, wherein the intensity of the green phosphomolybdenum complex measured spectrophotometrically at 695 nm provides the measure of total antioxidants found in the sample[32-34]. Phenolic compounds are widely and largely distributed in the plant kingdom which are known to show multiple biological functions including antioxidant activity[35]. Polyphenols in plant extracts react with specific redox reagents such as Folin-Ciocalteu reagent to form a blue complex that can be quantified by visible-light spectrophotometry[36-37]. The content values are estimated by total phenolic concentration equivalents of gallic acid, which is the most important polyphenol in natural products[37-38].

MATERIALS AND METHOD

Plant Collection and Extraction

Leaves of *P.grisea* were obtained from Mt. Kitanglad Mountain Range, Bukidnon, Philippines. Authentication of the plant's identity was done by the Department of Environment and Natural Resources forester Mr.SeroñoSehagun of the Mt. KitangladMountain Range Protected Area.In order to prepare the crude extracts, about 5.0 kg of the fresh samples of the leaves was properly washed in tap water and then rinsed in distilled water. The rinsed sample was then initially air dried for two weeks. The dried sample of the plant was pulverized using a sterile electric blender, weighed and percolated with enough 95% ethanol for three days. The solution prepared was filtered, concentrated *in vacuo* at temperatures not exceeding 40°C to give the crude ethanol extract. In hexane:water and chloroform:water solutions, 40 g of the crude ethanol extract was then sequentially partitioned. The hexane-, chloroform- and water-soluble portions was concentrated *in vacuo* and weighed to produce the hexane (PgH), chloroform (PgC) and aqueous (PgA) extracts, respectively. For the preparation of the plant decoction (PgD), about 2.0 kg of fresh and clean leaves were incised into pieces and boiled in sufficient amount of distilled water (1:2 ratio) for five minutes. The mixture was then filtered, cooled and stored in glass containers and freeze-dried.

Toxicity assay using brine shrimp lethality test

The toxicity test of the various crude extracts was tested against the brine shrimp *Artemia salina* leach following the protocol previously described by Meyer et al[6] and McLaughlin et al[7]with slight modifications. Four concentrations (1000-, 500-, 100-, and 10 ppm) were tested for each plant extract with three replicates. Podophyllotoxin and the solvent served as the positive and negative control respectively. The number of dead and alive nauplii were monitored, counted, and recorded after 6 and 24 hours. Examination of the results and the determination of the LC_{50} values were determined by employing the Reed-Muench method[39].

DPPH radical scavenging test

The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the P. grisea extracts were evaluated by comparison with the known antioxidant butylated hydroxytoluene (BHT) following the protocol as outlined by Lee and Shibamoto[40]. Each of the varying concentration of the extracts (500, 100, 50 and 25 µg/mL) was mixed with 3.0 mL of methanolic solution of DPPH (0.1 mM). Then, the mixture was vigorously shaken and allowed to stand at room temperature for one hour. Using the spectrophotometer, the absorbance was measured at 517 nm against methanol as blank. The percent of DPPH decoloration of the samples was computed based on the formula:

$$\text{Antiradical activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}$$

Total antioxidant capacity test by the phosphomolybdenum method

The evaluation of the total antioxidant capacity of the different P. grisea extracts were carried out using the phosphomolybdenum method described by Prieto et al[33]. Dispensed into 20 mL test tubes, 0.3 mL of the 200 µg/mL P. grisea extract solutions were separately added with 3.0 mL reagent solutions of 6M H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate. Then, the test tubes were incubated at 95°C for 90 minutes and then allowed to cool down at room temperature for the absorbance measurement at 695 nm using a spectrophotometer. The antioxidant activity was represented by ascorbic acid equivalents (water-soluble components) and BHT equivalence (fat-soluble components) that was derived from a linear equation established using ascorbic acid and BHT as reference standard. The results were recorded as means of three trials performed.

Total phenolics content test

The total phenolics of P. grisea extracts were determined employing the method described by Makkar et al[41] wherein 0.1 mL (0.5 mg/mL) of the extract was mixed with 2.8 mL of 10% Na_2CO_3 and 0.1 mL of 2N Folin-Ciocalteu reagent. The solution was allowed to stand for 40 minutes and the absorbance was measured at 725 nm. The total phenolic content was represented as milligrams of gallic acid equivalence per gram of sample. The calculations were based on the standard calibration curve derived from the various concentrations of gallic acid (25-200 ppm) and then the results were recorded as GAE (mg/g).

RESULTS

Toxicity assay using brine shrimp lethality test

Table 1 presents the mortality rates of the brine shrimp *A. salina* after 24 h exposure to different concentrations of the *P. grisea* leaf extracts as well as the concentration of the extracts that kills 50% of the brine shrimp (LC_{50}).

DPPH radical scavenging test

The ability of the *P. grisea* leaf extracts to scavenge the free radical DPPH are shown in Table 2.

Total antioxidant capacity by the phosphomolybdenum method

Illustrated in Figure 1 are the total antioxidant capacities of the *P. grisea* leaf extracts expressed as ascorbic acid equivalents (AAE) and butylatedhydroxytoluene equivalents (BHTE).

Total phenolicscontent

Figure 2 shows the total phenolics content of the various extracts of *P. grisea* leaves expressed as gallic acid equivalence (GAE).

Table 1: Toxicity of the various leafe xtracts of *P. grisea* to the brine shrimp *A. salina.*

LeafExtracts	PercentMortalityafter 24-h exposure* (%)				LC_{50} (ppm)
	10 ppm	100 ppm	500 ppm	1000 ppm	
PgE	8.57	31.03	62.07	84.85	290.07
PgH	00.0	5.71	84.38	100.00	281.84
PgC	51.35	69.09	91.18	100.00	< 10.00
PgA	10.00	33.33	80.08	100.00	199.53
PgD	0.00	3.08	23.91	65.85	817.52

* - mean of triplicate analysis
PgE *Prunus grisea* ethanol extract, PgH - hexane extract, PgC - chloroform extract,
PgA - aqueous extract, PgD decoction.

Table 2: DPPH radical-scavenging activities of *P. grisea* leaf extracts at various concentrations.

Extract	AntiradicalActivity, %*			
	25 ppm	50 ppm	100 ppm	500 ppm
PgE	11.50	19.28	22.84	52.67
PgH	8.81	12.43	13.81	23.12
PgC	10.97	17.78	18.90	33.55
PgA	0.00	0.44	5.31	32.24
PgD	32.99	88.22	91.47	92.85
BHT**	43.61	43.61	75.62	94.58

* - mean of triplicate analysis
** - butylated hydroxytoluene standard
PgE *Prunus grisea* ethanol extract, PgH - hexane extract, PgC - chloroform extract, PgA - aqueous extract,
PgD decoction.

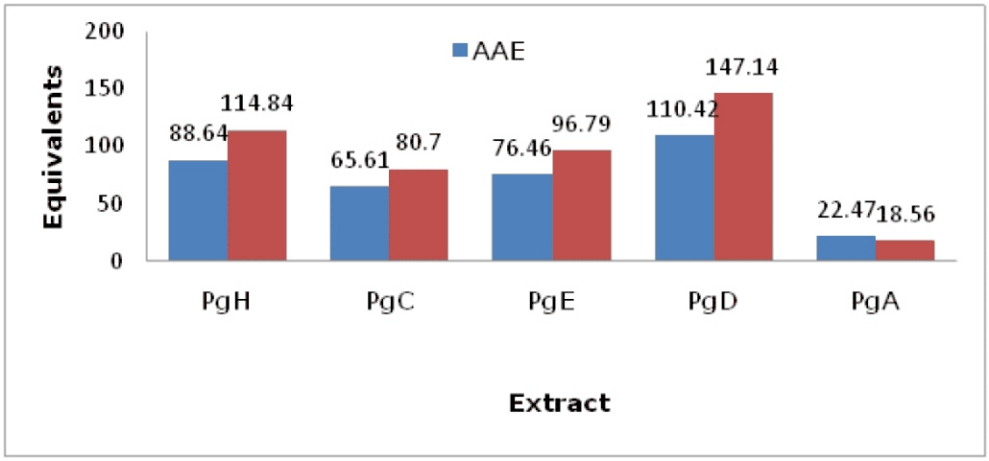

Figure 1: Total antioxidant capacities of *P. grisea* leaf extracts at 200-ppm concentration expressed as ascorbic acid equivalents (AAE) and buty. lated hydroxytoluene equivalents (BHTE)

DISCUSSION

Results show that the toxicity effects of the *P. grisea* leaf extracts on the test animal *A. salina* was concentration dependent. At 1000 ppm, PgH, PgC and PgA elicited a 100 percent mortality after a 24-hour exposure and by implication 1000 µg/ml of these *P. grisea* extracts is highly toxic to the brine shrimp larvae. At 10 ppm, PgC still exhibited a relatively high activity with 51.35% of the brine shrimps killed while the rest were non-toxic anymore. Crude extracts with LC_{50} values of less than 250 µg/ml are considered significantly active and have the potential for further investigation for the possible presence of bioactive components [6,42]. In reference to this, PgC and PgA extracts of *P. grisea* having the LC_{50} values of <10 ppm and

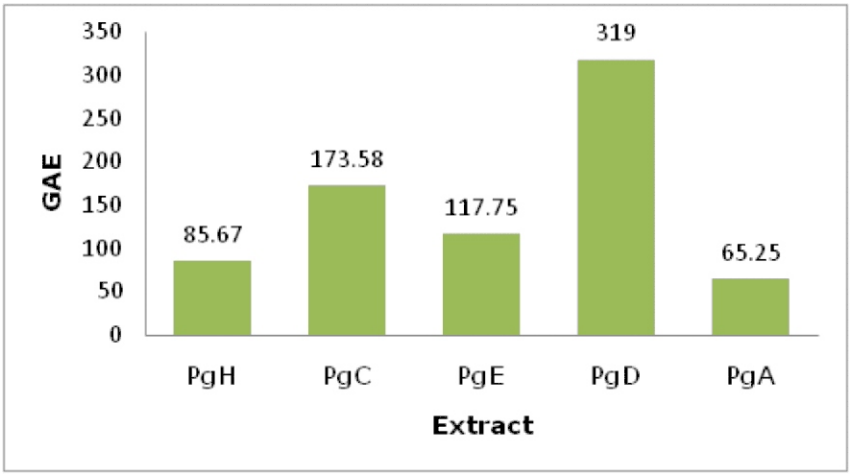

Figure 2: Total phenolics content of P. grisea leaf extracts at 500-ppm expressed as gallic acid equivalence (GAE)

199.53 ppm respectively, can be regarded to possibly contain cytotoxic properties.

As shown by the results, the radical-scavenging activity of all the *P. grisea* extracts increased as their concentrations were also increased. The decoction (PgD) exhibited greater activity than the control (BHT) at 50- and 100-ppm concentrations. However, it showed almost similar radical-scavenging ability as the control at 500 ppm. The results also indicate that the decoction of the leaves of *P. grisea* has high antioxidant activity compared with the hexane-soluble, chloroform-soluble, ethanol-soluble and aqueous extracts.

Meanwhile, all the extracts consistently showed the same order of antioxidant capacity in terms of both AAE and BHTE values i.e. PgD>PgH>PhE>PgC>PgA. The results also indicate that the top four extracts (PgD, PgH, PgE, and PgC) contain relatively more lipid-soluble antioxidants than water-soluble antioxidants.

The extracts displayed varying amounts of total phenolics content with the decoction (PgD) having the highest value of 319.00 GAE and the aqueous extract (PgA) having the lowest at 65.25 GAE. The high GAE value of PgD suggests the presence of substantial amount of phenolic compounds in this particular *P. grisea* extract which supports its the strong DPPH radical scavenging activity and its high total antioxidant capacity. Recent investigations have shown that many flavonoids and related polyphenols contribute significantly to the antioxidant activity of many fruits, vegetables and medicinal plants[43-45].

CONCLUSION

The study has shown that the different extracts of *P. grisea* leaves exhibited toxicity and antioxidant properties in varying degrees and amounts. In reference to the results of the brine shrimp lethality test, the chloroform (PgC) and aqueous (PgA) extracts of *P. grisea* leaves having LC_{50} values of <10 ppm and 199.53 ppm respectively, are considered significantly active and should be investigated further for the possible presence of cytotoxic components. Over-all evaluation of the results of the antioxidant assays strongly suggests that the decoction (PgD) has the most potent antioxidant properties among the extracts of *P. grisea* leaves. This indicates that bioactive molecules might be present in PgD which can be used as a prototype for development of new drugs and/or as a source of antioxidant pharmaceutical raw materials.

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