Research Paper

Antimicrobial activity of crude oil extracts from fruits of *Cynometra ramiflora* Linn and *Pandanus conoideus* Lamk from Papua New Guinea against ten pathogenic micro-organisms

Accepted 17th October, 2019

ABSTRACT

Plant-derived medicines are increasingly sought as alternatives to synthetic drugs. Adverse side-effects of antibiotics and resistance to certain pathogens are the reasons. This study investigated crude oil extracts of the fruits/seed kernels of *Cynometra ramiflora* and *Pandanus conoideus* and examined their efficacies against ten pathogens. The oils were assayed using disc (agar) diffusion technique against 8 bacteria, yeast and protozoa. The efficacies of the micro-organisms were evaluated by inhibition zones (IZs) and the potency of the oils were compared against antibiotics Gentamycin (10 µg) and Erythromycin (10 µg). Initially, 250 ml of crude oil was produced from 3.0 kg of pounded seeds of *C. ramiflora* while 500 ml of oil was extracted from 1.5 kg pericarps of *P. conoideus* using Soxhlet extractor and hot-water technique, respectively. The oils were designed in suspensions as: *C. ramiflora* 100% pure oil (CR), *P. conoideus* 100% pure oil (PC1), mixture of *P. conoideus* oil (10%) and palm oil (90%) (PC2) and mixture of *P. conoideus* oil (20%) and palm oil (80%) (PC3). Under sterile conditions, subcultured microbes (100 µL) were transferred onto agar plates. Then 40 paper discs (6 mm diameter) were impregnated with 25 µL of each oil suspensions. The impregnated discs were transferred to seeded media. Ten replicates were prepared for each oil suspensions and incubated at 30-37°C for 24-48 h. After incubation, the IZs were measured for each replicate twice and an average reading was noted. As per the results, pure oil of *C. ramiflora* indicated minimal efficacy (IZ 6 mm) against all test pathogens except for *Micrococcus luteus* with nil bioactivity. A maximum antibacterial activity was observed against *Salmonella typhimurium* (IZ 17 mm) equivalent to antibiotic Gentamycin (IZ 17 mm) but less effective than Erythromycin (IZ 30 mm). Alternatively, the oils of *P. conoideus* showed nil to low efficacy against ten pathogens. PC1 demonstrated minimal antibacterial activity (IZ 6 mm) against *Bacillus cereus*, *Escherichia coli* and *Neisseria gonorrhoeae* while it was ineffective against other seven micro-organisms. PC2 showed no potency against nine micro-organisms except for *N. gonorrhoeae* with minimal IZ (6 mm). PC3 was ineffective (IZ 0 mm) against ten micro-organisms screened.

Key words: crude oil extracts, *Cynometra ramiflora*, *Pandanus conoideus*, microbial susceptibility screening, pathogenic micro-organisms, inhibition zone, Papua New Guinea

INTRODUCTION

Plants continue to provide raw materials for many modern herbal and pharmaceutical drugs to treat various ailments and diseases as well as offer other health benefits. About 25% of prescribed drugs used globally are sourced from plants (Rates, 2001) and ca. 90% of all human diseases (bacterial infections, cancer, and
immunological disorders) are treated with natural products (Newman and Gragg, 2007). Particularly, in the developing world, traditional medicine (herbs) is an important part of livelihoods used by generations. FAO (2004) reported that 80% of the population in developing countries depend on medicinal plants for primary health care needs. In fact, modern drugs evolved from folk and traditional medicines (Morsy, 2014). Jain et al. (2019) added that plant-derived drugs were part of human evolution and healthcare for thousands of years. The use of *Pongamia pinnata* L and *Terminalia chebula* has a long history with Ayurveda, Siddha, and Unani systems of traditional medicine in India (Muthu et al., 2006; Bai, 2019). *Morinda citrifolia* is used for millennia as a panacea for wide range of ailments/afflictions in the Pacific (Sakulas et al., 2010). The main reasons for seeking herbal medicine are easy accessibility, affordability, limited supply of synthetic drugs in hospitals/pharmacies and the trust for the herbs. In addition, many are forced to seek herbal medicines due to adverse side-effects of many synthetic drugs and resistance of certain pathogens to antibiotics (Jain et al., 2019). Thus, numerous researches were pursued into medicinal plants for their phytochemistry and bioactivity in many parts of the world. For instance, >3,000 plant species were reported to have anti-cancer properties (Graham et al., 2000; Uddin et al., 2009) and >500 medicinal plants were discovered in Bangladesh (Uddin et al., 2009). Shrestha et al. (2015) recorded 33 medicinal plants in Nepal. Also, Indian studies found seed extracts of *Parkia javanica* to have anticancer effect (Khangemban et al., 2018) while fruit extracts of Algerian grown *Fraxinus excelsior* have antioxidant and antibacterial effects (Amamra et al., 2018).

In Papua New Guinea (PNG), traditional herbs for medicine provide primary health care needs for the bulk of the population. About 70% of population (from 8.5 million) lives in rural communities located in rugged/mountainous or inundated country where accessibility to infrastructures (road, transport, communication and health services) is often impossible. PNG, being a developing country, tropical ailments/diseases are prevalent and many rural communities rely on plant-derived medicines to treat these diseases. The first case study into PNG medicinal plants began in 1977 by Holdsworth. According to Woodley (1991), 127 medicinal plants belonging to 71 families were identified. Subsequently, Wau et al. (2010) reported antimicrobial activity of the fruit latex of *Ficus botryocarpa* and Zure et al. (2010) identified immunostimulant activity from the phytochemicals (alkaloids and isoflavonoids) of *Piper bolanicum*. Recently, Timi et al. (2018) found isolate of perennial plant *Euphorbia geniculata* to have antibacterial potency. It is anticipated that PNG’s high biodiversity forest ecosystem holds medicinal plants with huge potential for conventional drugs.

The plant species of interest in this research are crude oil extracts from the fruits of *Cynometra ramiflora* Linn and *Pandanus conoideus* Lamk. *C. ramiflora* (Fabaceae) is a small to medium-sized tree (Afrin et al., 2016) found in tidal and black-mangrove forests of the lowland forest up to 400 m above sea level (Useful Tropical Plants Website, 2018). The tree is distributed in the tropical regions of PNG, Indonesia (Asia) and Australia (Cooper, 2015). Many investigations of the species in Asia involved isolation of ingredients from the leaves and stem barks, phytochemical analysis and test for microbial efficacy (Afjalus et al., 2013). For instance, the authors evaluated bark extract for neuropharmacological, antibacterial and antinociceptive effects in Bangladesh. Also, a leaf extract synthesized iron oxide nanoparticles indicated effective antibacterial activity and stressed for developing antibacterial drug (Grois et al., 2016). Additionally, a leaf extract tried in Indonesia showed antiviral potency against Dengue virus (Meutia et al., 2017). However, very little work has been reported on seed (kernel) extracts and their bioactivity. Merca et al. (1989) isolated a lectin (highly acidic protein) from seeds while oil extracts from seeds were used to make lotion for skin diseases (Siraj et al., 2013; Afrin et al., 2016).

On the other hand, *P. conoideus* (Pandanaceae) is a monocot endemic to the island of New Guinea (Bourke, 2005; Rohman and Windarsih, 2018). It grows in tropical environments from lowlands up to 2000 m altitude (Stone, 1992) and commonly found near creeks. *P. conoideus* bears an edible red/orange and yellow fruit (pericarp); thus, it is commonly called red fruit (Indonesia) and marita (PNG). In Indonesia, red fruit has been used by Papua people as a natural remedy for variety of diseases (Wismandau et al., 2016; Achadiyani et al., 2016). In PNG, *P. conoideus* is cultivated in gardens for edible oil (Gusamo and Jimbudo, 2015) and culturally it is considered as a delicacy in the highlands region. Numerous studies on phytochemistry (characterization) and bioactivities of the oil extract are well documented based on Indonesian grown material (Rohman and Windarsih, 2018). Chemical analysis of the oil extract has high level of monounsaturated fatty acid (oleic acid), β-carotene, β-cryptoxanthin, α-tocopherol, phenolic compound and flavanoid (Mun’im et al., 2006; Khie et al., 2009; Mutingrum et al., 2012; Rohman et al., 2012; Salazar, 2018). The following health benefits were highlighted: strengthens body’s immune system and inhibits virus that triggers HIV/AIDS, heals/prevents spread of cancer, lowers blood pressure and prevents
stroke, provides sufficient insulin and prevents diabetes, prevents osteoporosis and improves brain functions (Khie et al., 2009; Rohman et al., 2012; Mun'im et al., 2006; Wismandanu et al., 2016; Salazar, 2018). Additionally, the oil treats hypertension, heart disease (arteriosclerosis) (Budi and Paimin, 2004); reduces obesity and increases lipolytic activity in adipose tissue, prevents age-related cognitive decline and dementia (Asensio et al., 2008) and malaria (Limbongan and Malik, 2009). Next, Indrawati (2016) reported minimal to maximum antibacterial activity (inhibition zones 8.28-32.0 mm) against common bacteria. Further, an acute toxicity study in rats indicated non-toxicity and categorized as Category 5 GHS (Globally Harmonized System for Classification Substances and Mixtures) (Wismandanu et al., 2016). Furthermore, Rohman and Windarsih (2018) reported that due to many functional health benefits, the oil is highly-priced (10-15 times) than other common oils (e.g. palm, corn, soybean, and canola) on the Indonesian market.

Disk (agar) diffusion is a standard method widely used for assaying plant extracts for antimicrobial activity (Perez et al., 1990) under a controlled laboratory condition. This test involves isolation of plant’s phytochemicals and screening the active ingredients against target micro-organisms for efficacy. The growth and proliferation, and the degree of the ingredients to inhibit the growth of the organisms are evaluated (Bauer et al., 1966). According to Prakash (2009) and Jacob et al. (2016), the diameter of inhibition zones of micro-organisms during microbial screening test are rated/classified as maximum (10-20 mm), moderate (10-15 mm) and minimal to no antimicrobial activities (<10 mm).

This study aimed to extract crude oils of Cynometra ramiflora and Pandanus conoides, and screened against strains of pathogenic micro-organisms (3 Gram-positive and 5 Gram-negative bacteria, 1 species each of protozoa and yeast). The antimicrobial susceptibilities (efficacies) of the oil extracts against the pathogens were ascertained by measuring inhibition zones using disc diffusion method. Fat content analysis, characterization, and phytochemistry of the fats were not part of the investigation.

**MATERIALS AND METHODS**

**Sampling and preparation of fruit samples**

More than 300 mature (ripe) fruits were collected from two trees of C. ramiflora growing in Lae, PNG. The hard coverings of fruits of C. ramiflora were removed (de-shelled) with a knife. More than 300 seed kernels were weighed for green (initial) weight and dried at room temperature for 2 days. The pre-dried seed kernels were re-weighed and milled (pounded) into 3000 g powdery form. For P. conoides, three ripe fruits (pericarps) were collected from Kimagi village, Kerowagi District, Simbu Province. The pericarps were stored in a cool room (refrigerator) for 10 days to prevent biodegradation. During storage, mold fungi growths were observed prior to extraction and experimentation.

**Aqueous extract of crude oils**

A Soxhlet extractor was used to extract crude oil of C. ramiflora. A wad of cotton wool was plugged in the hole of the extraction unit. The 3000 g ground sample was placed in the extraction unit. A clean/dried round-bottom flask was weighed and attached to the extraction unit. The extraction unit was filled with hexane (3730-2 SL. Class 3, Minimum Assay 98.0%, Banksia Scientific Co., Australia) to siphon once and half-filled with hexane again. The condenser unit was attached and heated on the electric heating mantle and heat was regulated to permit condensation of 5-10 drops per second. The extraction process was allowed for 8 h. The flask was then removed and the hexane was distilled to separate the oil. Finally, 20 ml of oil was retained in the flask.

On the other hand, hot water extraction technique was used to extract oil from P. conoides fruits. Firstly, the fruits were washed with water to remove foreign matter. The fruits were weighed (2000 g), de-cored, chopped with the pericarps intact (1500 g) and boiled in water (1:1 ratio) until tender. The boiled pericarps were removed and cooled at ambient temperature. The same water used for boiling was added (1:1 ratio) to the pericarps and pulped (macerated) by squeezing with gloved-hands. The seeds were discarded and a thick creamy substance (puree) was collected. The puree was filtered to remove any foreign particles, heated for a few minutes and allowed the concentrate to cool overnight. The creamy concentrate was then separated into a layer of water and thick creamy oil. The creamy oil was dispensed and centrifuged at 3000 rpm for 30 min. The top layer oil was siphoned off as crude oil producing 150 ml while the rest was a puree.

**Mixing oil extracts with palm oil as carrier solvent**

The oil extract of C. ramiflora was pure (100%) oil (CR) intended to use in microbial test without mixing with palm oil as a carrier solvent. Except for P. conoides, the
oil extracts were mixed with palm oil. The crude oils were mixed in three 60 ml bottles and the composition of the mixtures were: (a) 100% pure P. conoideus oil (PC1), (b) 10% P. conoideus oil to 90% palm oil (PC2) and (c) 20% P. conoideus oil to 80% palm oil (PC3).

**Bioassay: Preparation of micro-organism, assay plates and paper discs**

Pure cultures of ten micro-organisms (Table 1) were prepared in bottles and stored in the fridge. The ten indicator micro-organisms selected and screened were pathogens responsible for inducing various diseases in humans and animals.

Nutrient agar (11.3 g) was added in distilled water (400 ml) and sterilized in an autoclave at 115°C for 20 min. After sterilization, nutrient agar (15-20 ml) was dispensed into ten sterile plastic Petri-discs (standard size 100 mm internal diameter). The Petri-discs (with agar) were left to cool and solidify at room temperature prior to bioassay. Paper discs (6 mm diameter) were cut from Matricel filter paper (1 mm thick) with a paper-hole puncher and were sterilized.

**Microbial test**

A standard disc (agar) diffusion technique was employed to conduct the microbial susceptibility screening test (Bauer et al., 1966). Onto the ten prepared agar plates, 100 µL of ten subcultured microbes were transferred with a sterile micropipette and spread evenly using a glass hockey rod under sterile conditions. The glass hockey rod slightly dipped in ethanol solution (99%) and heated over the Bunsen burner flame for a few seconds (2-5 s) and removed from the flame to cool. The microbes were streaked evenly over the Petri-discs with glass hockey rod. Using a micropipette, 40 paper discs were impregnated with 25 µL suspensions of CR, PC1, PC2, and PC3 and dried. A total of 10 replicates per oil suspension were prepared. The impregnated discs were then transferred to seeded media using a sterilized stabbing needle, which was held over the flame to red hot occasionally before use. The discs were pressed gently to ensure firm contact with the seeded media. The yeast (Candida albicans) and protozoa (Trichomonas vaginalis) were placed in an incubator at 30°C whilst the bacteria were incubated in a separate incubator at 37°C. The incubation of microbes took 24 to 48 h to facilitate maximum growth. After incubation, the zone of inhibition (mm) was measured for each replicate using a ruler. Each measurement of the inhibition zone was taken twice and an average reading was recorded (Table 2).

In addition, two synthetic antibiotics weighing 10 µg each of Gentamycin and Erythromycin were used as standard/reference substances in the experiment. The preparation and test of antibiotics against the microorganisms followed the same procedure described above. The bioactivities exhibited by two antibiotics were compared with that of crude oil extracts (CR, PC1, PC2, and PC3) of the two test species (Table 2).

**RESULTS AND DISCUSSION**

Different efficacy levels of the oil extracts of two test species as well as two reference antibiotics on the ten indicator micro-organisms are shown in Table 2. The bioactivity displayed by the ten micro-organisms was indicated by clear zones of inhibition (IZ) in millimeter (mm). In this case, the higher the IZ, the higher the efficacy of the oils against the growth of micro-organisms.

According to the results (Table 2), oil extracts (CR, PC1, PC2 and PC3) of the fruits of two test species demonstrated variability in their antimicrobial activity against the ten micro-organisms screened. The antimicrobial activities were determined by their IZs and rated according to descriptions provided by Prakash (2009) and Jacob et al. (2016). The IZs (efficacies) of the oil extracts were then compared with standard (synthetic) antibiotics (Gentamycin (GM) and Erythromycin (EM)). The antibiotics (GM and EM) indicated high efficacies against the micro-organisms. For instance, the IZ of GM and EM ranged between 15-24 and 6-30 mm respectively. In particular, EM revealed minimal

<table>
<thead>
<tr>
<th>Strains of micro-organisms</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria (gram-positive)</td>
<td><em>Bacillus cereus, Micrococcus luteus, Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Bacteria (gram-negative)</td>
<td><em>Escherichia coli, Klebsiella pneumoniae, Neisseria gonorrhoeae, Proteus vulgaris, Salmonella typhimurium</em></td>
</tr>
<tr>
<td>Protozoa</td>
<td><em>Trichomonas vaginalis</em></td>
</tr>
<tr>
<td>Yeast</td>
<td><em>Candida albicans</em></td>
</tr>
</tbody>
</table>
IZs (6 mm) for *Proteus vulgaris* (G-) and yeast (*C. albicans*) whilst it was significantly effective against *Escherichia coli* (G-) and *Salmonella typhimurium* (G-) with IZs (28 and 30 mm) respectively. On the other hand, GM proved moderate to high efficacy against all micro-organisms screened.

**Bioactivity of *Cynometra ramiflora* oil extracts**

The oil extract of *C. ramiflora* (CR 100%) showed nil to minimal efficacy/potency (IZ 0-6 mm) for ten strains of pathogens screened except for *Salmonella typhimurium* (G-). It was ineffective against *Micrococcus luteus* (G+) (IZ 0 mm) while a minimum antimicrobial activity (IZ 6 mm) was observed for nine micro-organisms tested apart from bacterium *S. typhimurium* (G-). Additionally, it was noted that CR oil indicated antimicrobial activity similar to that of antibiotic EM (IZ 6 mm) against bacterium *P. vulgaris* (G-) and yeast *C. albicans*. Further, an interesting observation was made for CR oil, which exhibited a maximum efficacy (IZ 17 mm) against bacterium *S. typhimurium* (G-). Comparatively, the antibacterial activity demonstrated by CR oil was comparable (equivalent) to IZs of antibiotics GM (17 mm) and EM (30 mm) for the same bacterium. Similar observations (effective antibacterial activities) were reported by Afjalous et al. (2013) and Grois et al. (2016) from the bark and leaf extracts of *C. ramiflora*, respectively. The finding of this case study indicates that oil extract of seed kernels of *C. ramiflora* holds potential for developing an antibacterial drug to treat diseases inflicted by *S. typhimurium* (e.g. Gastroenteritis (gut inflammation), Diarrhoea, Vomiting, Fever, Abdominal cramps) (https://www.yourgenome.org/facts/what-is-salmonella, 2018).

**Bioactivity of *Pandanus conoideus* oil extracts**

The oil extracts of *P. conoideus* in three different forms (PC1, PC2 and PC3) indicated nil to minimal antimicrobial activity against ten micro-organisms tested. A minimal antibacterial activity with IZ (6 mm) was observed for PC1 (100%) when screened against three bacteria *Bacillus cereus* (G+), *E. coli* (G-) and *Neisseria gonorrhoeae* (G-) whilst it was ineffective against seven other micro-organisms (IZ 0 mm). For the case of PC2 (mixtures of *P. conoideus* (20%) and palm oil (80%)), it showed minimal antibacterial activity with IZ (6 mm) against bacterium *N. gonorrhoeae* (G-). The oil was ineffective against nine other micro-organisms (IZ 0 mm). Moreover, PC3 (mixtures of *P. conoideus* (10%) and palm oil (90%)) were significantly ineffective against ten strains of micro-organisms screened with IZ (0 mm). The results of this research contradict the findings of Indrawati (2016), who reported minimal to maximum antibacterial activity. The microbial efficacy of the *P. conoideus* oil was nil to minimal and incomparable to standard antibiotics used in this study. Also, it was observed that increasing the amount of palm oil (%) as a carrier solvent in the mixture with *P. conoideus* oil decreased the efficacy against the micro-organisms. Therefore, the results of this case study implied that oil

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**Table 2: Antimicrobial activity of crude oils of fruits of *C. ramiflora* and *P. conoideus* and reference substances against ten micro-organisms.**

<table>
<thead>
<tr>
<th>Micro-organisms</th>
<th>CR</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>GM</th>
<th>EM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em> (G+)</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em> (G+)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (G+)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (G-)</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>28</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (G-)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em> (G-)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> (G-)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> (G-)</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td><em>Candida albicans</em> (Y)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td><em>Trichomonas vaginalis</em> (Pz)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>20</td>
</tr>
</tbody>
</table>

G+: gram positive bacteria, G-: gram negative bacteria, Y: yeast, Pz: protozoa, CR: pure *C. ramiflora* oil, PC1: pure *P. conoideus* oil, PC2: mixture of *P. conoideus* oil and palm oil, PC3: mixture of *P. conoideus* oil and palm oil, GM: Gentamycin, EM: Erythromycin.
extracts of *P. conoideus* have poor antimicrobial phytochemicals. The low efficacy of *P. conoideus* oil could be due to the onset of rancidity. There was a delay (10 days) between harvesting of the fruits and experimentation. Thus, the active ingredients of the fruits were suggested to have undergone partial oxidation/hydrolysis upon exposure to the environment and/or biodegraded via the actions of mould fungi. Also, the low efficacy could be attributed to thermal degradation of volatile compounds during hot water extract/heating extraction (Das et al., 2010).

### CONCLUSION

The crude oil extracts of *C. ramiflora* showed minimal antimicrobial activities against nine micro-organisms screened while a high (maximum IZ) antibacterial efficacy was observed against *Salmonella typhimurium* (G-). The IZ (mm) demonstrated by *C. ramiflora* against the same bacterium was comparable (equivalent) to synthetic antibiotics tried in the experiment. The finding indicates the potential of the oils for developing an antibacterial drug for *Salmonella typhimurium* (G-) infections. Further research on the analysis of phytochemical constituents (including acute toxicity test) of oil extract of the seeds for pharmaceutical and therapeutic purposes is recommended.

In contrast, oil extracts of the pericarps of *P. conoideus* were ineffective (low to minimal antimicrobial activity) against all micro-organisms screened. PCI (pure oil extract) indicated minimal antibacterial activity. The finding showed that oil extract of pericarps of the *P. conoideus* had no antimicrobial activity under the conditions used. To validate the findings of Indrawati (2016), a repeat bioassay test using fresh *P. conoideus* fruits immediately after harvest to avoid rancidity is recommended. Also, the use of polar (methanol) and non-polar (hexane) solvents for crude oil extraction instead of hot water/heat extraction to exclude thermal degradation of active (volatile) compounds is desirable.

### ACKNOWLEDGEMENTS

Kind assistance was provided by Reilly Nigo, Wap Kuipa, Nehemiah Pelis Wangiwan and Richard Laki in the laboratory experiments, Applied Science Department, PNG University of Technology. Mr. Artis Vinas of the Forestry Department, PNG University of Technology identified the plant species. Dr. Anthony Harakufe of Roots Organic Inc commented on the manuscript. While Professor Barbara Ozarska and Dr. Benoit Belleville of the University of Melbourne, Australia, read the paper.

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