Triterpenoids from *Agathis robusta* Aerial Parts and Their Hepatoprotective Activity

Amal H. Ahmed*, Shaza A. Mohamed

**ABSTRACT**

**Objective:** While *Agathis robusta* contains important phytochemical constituents and has been linked to a variety of biological activities, there is currently insufficient research on the plant’s total phytochemical constituents or pharmacological activity. **Materials and Procedures:** The aerial part of *Agathis robusta* was extracted with 70% methanol and was screened for new phytochemical components. The structures of the isolated compounds were elucidated by spectroscopic data interpretation. The hepatoprotective activity of the isolated compounds was investigated. **Results:** Four known triterpenoids and two new compounds were isolated for the first time from the methanolic extract of the aerial parts of *A. robusta*. **Conclusion:** For the first time, new triterpenoidal saponins with high hepatoprotective activity have been isolated from the aerial portion of *A. robusta*. As a result, it is suggested that more emphasis be placed on this plant’s biological behavior.

**Key words:** *Agathis robusta*, Triterpenoid saponins, Hepatoprotective activity.

**INTRODUCTION**

The coniferous tree *Agathis robusta* (Common Name: Queensland Kauri) belongs to the Araucariaceae family. It is a large evergreen conifer with a slow growth rate but a long-life span that can grow to a height of 25-30 meters, with a maximum height of 43 metres. In forest-grown trees, the crown is dense, but it can also be thin, and it becomes more elongated as the tree matures. The straight, cylindrical bole may be free of branches for more than half the tree’s height and 100-150 cm or more in diameter. *Agathis robusta* tree is growing in a seasonal tropical climate in north Queensland producing late wood during cooler and drier periods. Glycosides, tannins, flavonoids, saponins, carbohydrates, fixed oil, and mucilage were discovered in the leaves of *A. robusta*.

Agathisflavone, 7'-O-methyl-agathisflavone, cupressuvflavone, rutin and shikimic acid, were isolated from the ethanolic extract of the aerial parts of *A. robusta*.

The oleo-resin of *Agathis robusta* had found to contain the two known diterpene acids, levopimaric and communic acids, hydrodistilled resin and leaf essential oils were analysed, and 34 constituents (98.2 percent of the resin oil) and 43 constituents (91.2% of the leaf oil composition) were discovered. Isobornyl acetate (37.9%), myrtenol (5.8%) were the major constituents of the resin oil, while -selinene (18.1%), rimuene (14.2%), and caryophyllene (5.8%) were included in the leaf oil. The leaves’ methanolic extract has good antimicrobial properties, and the essential oil has an intriguing antimicrobial impact.

No sufficient work is present about either the total phytochemical constituents of *A. robusta* plant or the pharmacological activities so, the aim of this study is to isolate new compounds from the plant and investigate some of its biological activities.

**MATERIALS AND METHODS**

**Plant material**

Plant material was collected at May 2018 from Mohammed Ali Museum, Giza, Egypt & identification of the plant material was confirmed by Dr. Trease Labeb, senior specialist of plant taxonomy, Orman Garden, Giza, Egypt as well as by comparison with reference herbarium specimens. A voucher specimen (code AR-1518) has been deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.

**Extraction and isolation**

Aerial parts of *Agathis robusta* (6 kg) were extracted with 70% methanol (MeOH) (20 L x 3) three times at room temperature. The concentrated methanolic extract (850 gm) were suspended in 500 ml distilled water and then extracted successively with ethyl acetate (Et OAc) (3 L x 3) and n- butanol (n-BuOH) (2.8 L x 3), and concentrated to afford the residues of Et OAc fraction (180 gm), n-BuOH fraction (260 gm) and residual aqueous fraction (372 gm), respectively.

The Et OAc extract (180 gm) were applied to a silica gel column chromatography (c.c.) and eluted with mixtures of n-hexane-Et OAc of increasing polarities to afford 10 fractions (1 to 10). Fraction-1 (660 mg) was subjected to another silica gel column chromatography and eluted with n-hexane-EtOAc (1:1) to give six fractions (1.1 to 1.6, a purified compound 3 was obtained from the fraction 1.2).

Cite this article: Ahmed AH, Mohamed SA. Triterpenoids from *Agathis robusta* Aerial Parts and Their Hepatoprotective Activity. Pharmacogn J. 2022;14(4): 362-366.
Fraction 1.4 was further fractionated on silica gel column chromatography and eluted with n-hexane- EtOAc (6:1) to yield 12 subfractions (named 4.1 to 4.12). Subfraction 4.8 (30 mg) was subjected to sephadex c.c. and eluted with (MeOH: H₂O, 7:1) to give three fractions (A to C). Purified compounds 1 & 2 were isolated from fraction B & C respectively.

The n-ButOH extracts (260 gm) were chromatographed on silica gel column chromatography (10 × 12 cm), eluted with CHCl₃-MeOH mixtures of increasing polarity (9:1 → pure methanol) to afford 20 fractions (1 to 20).

Compound 4 was isolated from fraction 9 (1.3 g). Fraction 13 (1.5 g) was further chromatographed by column chromatography and eluted with chloroform: methanol (CHCl₃-MeOH, 8:3) from which a purified compound 5 was isolated.

Fraction 16 (2.8 g) was purified by sephadex c.c. eluting with (MeOH-H₂O, 3:2) to give four fractions (a to d), a purified compound 6 was isolated from fraction d.

**General experimental procedures**

**NMR Spectroscopy:** NMR analysis was done using Bruker spectrometer operating at 400 MHz for 'H & at 100 MHz for 13C. All samples were prepared in DMSO-d₆ with TMS as an internal reference. Chemical shifts represented in ppm and coupling constant J in Hertz.

**Electrospray Mass Spectroscopy** (ESI-MS) was carried out using Thermo Finnegan LCQ: Advantage MAX (ion trap) instrument (Finnegan, Bremen, Germany). Samples dissolved in 10ul 50% methanol. (The analysis was done in NMR unit, Faculty of Pharmacy, Cairo University, Egypt).

**UV Spectrophotometer:** Shimadzu, UV 240 was used for recording different UV spectra.

**Hepatoprotective study in HepG2 cell line**

(Done at the Regional Center for Mycology & Biotechnology, Al-Azhar University)

**Principle**

HepG2 Cell lines are suitable for in vitro model system for the study of polarized human hepatocytes. HepG2 cell line with proper culture conditions displays robust morphological and functional differentiation with a controlled formation of apical and basolateral cell surface domain in models. The HepG2 cells were exposed to toxicant containing 1% CCl₄ along with /without tested sample of different concentrations or the medium alone is considered as control. At the end of the period, cytotoxicity was assessed by estimating the viability of the HepG2 cells by the MTT reduction assay.

**Methods**

The HepG2 cells of human liver cell line was cultured in DMEM (Dulbecco’s modified eagle’s medium) contains 10% fetal calf serum, penicillin (100 U) and streptomycin (100µg).

Hepatoprotective effect in HepG2 cell line estimated by MTT Assay. The monolayer cell culture was trypsinated and the cell count was adjusted to 1.0 x10⁵ cells/mL using medium containing 10% newborn calf serum. To each well of the 96-well microtitre plate, 0.1 mL of the diluted cell suspension formed, the supernatant was flicked off, the monolayer was washed once, and 100µL samples with various drug concentrations were added to cells in wells of the microtitre plate. The plate was then incubated at 37°C in 5% CO2 atmosphere for 24 h.

**Experimental design**

Human liver HepG2 cells were exposed to a medium containing CCl₄ (1%) with/without various concentrations from the tested compounds (6.25, 12.5, 25, 50, 100 and 200 µg/mL). Then, cytotoxicity was assessed by estimating the viability of HepG2 cells by MTT reduction assay.

**The experimental groups were as follows:**

Group 1: Control, untreated HepG2 cell line
Group 2: HepG2 cells with 1% CCl₄
Group 3: HepG2 cells with 1% CCl₄ and tested compound 6 (C6)
Group 4: HepG2 cells with 1% CCl₄ and tested compound 6 (C6)
Group 5: HepG2 cells with 1% CCl₄ and silymarin standard drug

Each treatment was repeated four times (i.e., 4 wells for each treatment).

**MTT assay**

Following treatment with the above-mentioned methods, after 24 h incubation, the medium was removed and 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml; Sigma, St. Louis, CA, USA) was added to each well. The plates were gently shaken then incubated in the dark at 37°C for an additional 4 h in 5% CO₂ atmosphere. The reaction was stopped by the addition of 150 µl DMSO (Sigma) and the absorbance of samples at 570 nm was measured with a microplate reader (SunRise, Tecan, USA).

The tetrazolium salt (3-(4, 5–dimethylthiazole-2-yl)-2, 5 diphenyl tetrazolium bromide, MTT) is introduced into cells and reduced in mitochondria dependent reaction to yield a blue colored formazan product. The product accumulates within the cell due to the fact that it cannot pass through the plasma membrane. On solubilization of the cells, the product is liberated and can be readily detected and quantified by a simple colorimetric method. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity, which in turn maybe interpreted as a measure of viability and/or number of cells. The assay has therefore been adopted for use with cultures of exponentially growing cells. Determination of the cell ability to reduce MTT provides a measure of the cell’s ability to survive after exposure to test compounds shows hepatoprotective effect. The optical density of the formazan formed in the control cells was taken as 100%. The viability of HepG2 cells in other groups was presented as a percentage of the control cells.
Forty-eight animals (48) were divided into 8 groups, 6 rats in each as follows:

**Group I:** Rats served as negative control and were orally administered normal saline for 21 days.

**Group II:** Rats were orally administered compound 5, solubilized in distilled water (1 mg/kg body weight) for 21 days.

**Group III:** Rats were orally administered compound 6, solubilized in distilled water (1 mg/kg b.wt.) for 21 days.

**Group IV:** Rats served as positive control and were orally administered normal saline for 21 days.

**Group V:** Rats were orally administered silymarin (25 mg/kg b.wt.) as a reference hepatoprotective drug for 15 days followed by administration of CCl4 (1.5 mg/kg b.wt., intraperitoneal) twice weekly until day 21.

**Group VI:** Rats were orally administered 70% dried methanol extract of CCl4 (1.5 mg/kg b.wt., intraperitoneal) twice weekly until day 21.

**Group VII:** Rats were orally administered compound 6, solubilized in distilled water (100 mg/kg b.wt.) for 15 days followed by administration of CCl4 (1.5 mg/kg b.wt., intraperitoneal) twice weekly until day 21.

**Group VIII:** Rats were orally administered compound 6 solubilized in distilled water (1 mg/kg b.wt.) for 15 days followed by administration of CCl4 (1.5 mg/kg b.wt., intraperitoneal) twice weekly until day 21.

**Experiment design**

At the end of the experiment, the rats of all groups were anesthetized and blood samples were collected directly from retro-orbital plexus. The blood samples were allowed to clot for 20-30 min. Serum was separated by centrifugation at 37°C and used for estimation of various biochemical parameters. ALT (alanine aminotransferase) and AST (aspartate aminotransferase) activities in serum were determined according to Reitman & Frankel.  

**RESULTS AND DISCUSSION**

From the methanolic extract of *Agathis robusta* (F. Araucariaceae), four known compounds (C1 to C4) and two new triterpenoid saponins (C5 and C6) were identified.

Spectral evidence, especially NMR and mass spectra, led us to postulate the isolated compound structures. Oleanolic acid was identified after acid hydrolysis as an aglycone of saponins 1, 2, 3, 4 and 6, while hederagenin was identified by acid hydrolysis as the aglycone of saponin 5.

On the basis of spectral data, especially 1D and 2D NMR and MS and through comparison with the data in the literature, 15-18

Compounds 1 (C1): is identified as: 3-O-[α-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyleolic acid.15

Compounds 2 (C2): 3-O-[α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)]-[α-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyleolic acid. 15

Compounds 3 (C3): 3, 23-dihydroxy-12-oleanen-28-oic acid, 3-O-[α-L-rhamnopyranosyl-(1→2)]-α-L-arabinopyranosyl-28-O-[α-L-rhamnopyranosyloleanolic acid-17

Compounds 4 (C4): 3, 23-dihydroxy-12-oleanen-28-oic acid, 3-O-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-28-O-[α-D-glucopyranosyleolic acid]. 17

Compound 5 (C5) was obtained as an amorphous powder. The mass spectrum (negative ion mode) showed a quasimolecular ion peak at m/z 1073 ([M-H]-), indicating a molecular weight of 1074 with a suggestive molecular formula C_{58}H_{90}O_{14}. Other significant peaks were observed at m/z 911 ([M-H-162]-, 765 ([M-H-162-146]-, 749 ([M-H-162-162]-), 603 ([M-H-162-162-146]- and/or [M-H-162-162-162]- and 471 ([M-H-162-162-162-146]-132].

Based on these findings, the removal of two galactopyranosyl moieties, one rhamnopyranosyl, and one arabinopyranosyl to generate aglycone at m/z 471 (hederagenin-H = [C_{30}H_{48}O_{4}-H] = [C_{30}H_{47}O_{4}]) in accordance with the arabinose, rhamnose, and galactose sugar.

The peak at m/z 911 ([M-H-galactopyranosyl]) indicates a terminal galactopyranosyl unit. Additional peaks at 765 ([M-H-galactopyranosyl-rhamnopyranosyl]) = m/z 911-rhamnopyranosyl and 749 ([M-H-galactopyranosyl-galactopyranosyl]) = m/z 911-galactopyranosyl indicated a branched sugar chain with a disubstituted arabinopyranosyl moiety.17

Anomeric proton signals at 4.33 (d, J = 7.1 Hz, H-1), 6.17 (brs, H-1), 5.08 (d, J = 7.9 Hz, H-1), and 5.46 (d, J = 7.8 Hz, H-1) and carbon signals at 105.09, 101.67, 106.97, and 106.90, respectively, can be seen in the HMBC spectrum.

For each sugar unit, the 1 H- 1 H-COSY, TOCSY, HMQC, and HMBC spectra achieved full 1 H and 13 C chemical change assignments. The signals at 122.76 (CH-12) and 144.95 (C-13) in the 13C-NMR were seen in association with the signals at 122.76 (CH-12) and 144.95 (C-13).
Values were expressed as mean ± standard deviation in each group.

Table 1: In-Vitro Hepatoprotective Activity of C5 and C6 on HepG2 Cell line.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hepatoprotective effect (EC50) μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5</td>
<td>&lt; 5000</td>
</tr>
<tr>
<td>C6</td>
<td>&lt; 5000</td>
</tr>
<tr>
<td>Silymarin (Standard drug)</td>
<td>150</td>
</tr>
</tbody>
</table>

Table 2: In-Vivo Hepatoprotective Activity (The changes of ALT and AST levels).

<table>
<thead>
<tr>
<th>Group No.</th>
<th>ALT</th>
<th>AST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>11.13±1.12</td>
<td>22.60 ± 2.21</td>
</tr>
<tr>
<td>Group II</td>
<td>60.11±2.1</td>
<td>92.73 ± 2.7</td>
</tr>
<tr>
<td>Group III</td>
<td>40.1 ± 3.3</td>
<td>46.1 ± 2.2</td>
</tr>
<tr>
<td>Group IV</td>
<td>48.1 ± 3.1</td>
<td>45.7 ± 2.9</td>
</tr>
<tr>
<td>Group V</td>
<td>49.07 ± 3.93</td>
<td>28.4 ± 1.5</td>
</tr>
<tr>
<td>Group VI</td>
<td>30.6 ± 2.03</td>
<td>28.44 ± 1.55</td>
</tr>
<tr>
<td>Group VII</td>
<td>31.9 ± 2.2</td>
<td>27.87 ± 2.25</td>
</tr>
<tr>
<td>Group VIII</td>
<td>30.9 ± 3.2</td>
<td>28.44 ± 1.58</td>
</tr>
</tbody>
</table>

As an aglycone, NMR spectra revealed a pentacyclic triterpenoid skeleton from olean-12-ene. The cross peaks detected in the HMQCC spectrum indicating a relationship between the signals at δ H 5.48 (H-12) and C1 indisputably rendered the chemical change assignments of CH-12 and CH-3 for 1H and 13C spectrum indicating a relationship between the signals at δ H 5.48 (H-12) and C1 indisputably rendered the chemical change assignments of CH-12 and CH-3 for 1H and 13C.

CONCLUSION

In the present article two new compounds (C5& C6) and four known compounds (C1- C4) had obtained from the aerial parts of Agathis robusta for the first time, the new isolated compounds (C5& C6) showed strong in-vitro and in-vivo hepatoprotective effect.

DISCLOSURE STATEMENTS

No potential conflicts of interest were reported by the authors.

FUNDING

No funding.

REFERENCES

Ahmed AH, et al.: Triterpenoids from Agathis robusta Aerial Parts and Their Hepatoprotective Activity


GRAPHICAL ABSTRACT

ABOUT AUTHORS

Assoc. Prof. Dr. Amal Hussein Ahmed (6/03/1971) received her Ph.D. in 2001 from Al Azhar University. She Works as lecturer then as Associate professor of Pharmacognosy, Department of Pharmacognosy & medicinal Plants, Faculty of Pharmacy (Girls), Al Azhar University. She has several publications in peer-reviewed scientific journals including several research areas of chemistry of natural plants. In addition, to her contribution in national and international conferences. She supervised many PhD theses and students’ graduation projects. In addition to most of the academic activities.

Shaza Abdelhalim Mohamed, Associate professor at Pharmacognosy and Medicinal Plants Department, Faculty of Pharmacy (Girls), Al- Azhar University. She worked as lecturer at Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA) and at Faculty of Pharmacy, Russian University, Egypt. Also worked as assistant professor and coordinator at Faculty of Pharmacy, Taif University, KSA. Her B.Sc. degree was granted from Faculty of Pharmacy, Cairo University, while M.Sc. and Ph.D. (Pharmacognosy) degrees were granted from Faculty of Pharmacy (Girls), Al- Azhar University.