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Research Article

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

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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.

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INTRODUCTION

The coniferous tree *Agathis robusta* (Common Name: Queensland Kauri) belongs to the Araucariaceae family.[1] 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.[2] In forest-grown trees, the crown is dense, but it can also be thin, and it becomes more elongated as the tree matures.[3]

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.[4] *Agathis robusta* tree is growing in a seasonal tropical climate in north Queensland producing late wood during cooler and drier periods.[5] Glycosides, tannins, flavonoids, saponins, carbohydrates, fixed oil, and mucilage were discovered in the leaves of *A. robusta*.[6]

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

The oleo-resin of *Agathis robusta* had found to contain the two known diterpene acids, levopimaric and communic acids.[8] 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%), limonene (12.3%), bornyl acetate (7.4%), and 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 anti-inflammatory properties, and the essential oil has an intriguing antimicrobial impact.[9]

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 × 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 × 3) and n- butanol (n-BuOH) (2.8 L × 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).

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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 sephadix c.c. and eluted with (MeOH: H_2O, 7:1) to give three fractions (A to C). Purified compounds 1& 2 were isolated from fraction B& C respectively.

The n-BuOH extracts (260 gm) were chromatographed on silica gel column chromatography (10 × 12 cm), eluted with $CHCl_3$-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 ($CHCl_3$-MeOH, 8:3) from which a purified compound 5 was isolated.

Fraction 16 (2.8 g) was purified by sephadix c.c. eluting with (MeOH-H_2O, 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 ^{1}H& at 100 MHz for ^{13}C. All samples were prepared in DMSO-d_6 with TMS as an internal reference. Chemical shifts represented in ppm and coupling constant J in Hertz. (The analysis was done in NMR unit, Faculty of Pharmacy, Cairo University, Egypt).

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**.**

The two-dimensional NMR was recorded on a Bruker High Performance Digital FT-NMR Spectrometer, Advance III 400 MHz, using TMS as internal standard. The δ-values are reported as ppm relative to TMS and J-values in Hz.

Column chromatography (CC) was carried out on silica gel (Si_{60} F_{254}, 230-400 mesh, Merck). Pre-coated plates of silica gel $_{60}$ F_{254} were used for analytical purposes. Compounds were visualized under UV radiation (254, 365 nm) and by spraying plates with 10% Methanol/ Sulphuric acid, Naturstoff and 5% $FeCl_3$. All other solvents used for extraction and separation processes were of analytical grade (El-Nasr Chemicals Co., Abou- Zaabal, Egypt).

Acid hydrolysis of compounds: Few milligrams of each of the pure isolated compound were refluxed with 2 N HCl (2 h, 100° C), the hydrolysate mixture after neutralization with diluted solution of $NaHCO_3$ was extracted with chloroform in a separating funnel to separate the aglycone in the organic phase, while the sugar being in the aqueous phase.[10] The aqueous layer then filtered, concentrated and compared with standard sugars on paper chromatography using solvent system; n-butanol/acetic acid/water; 4:1:5. Spots were detected by spraying with a solution of aniline phthalate.

HEPATOPROTECTIVE STUDY IN HEPG2 CELL LINE[11-13]

(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% CCl_4 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 CCl_4 (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 5 (C5)

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 a 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 may be 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 to the formazan derivative 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.

Data analysis

The results were expressed as:

The percentage of viability was calculated as [(ODt/ODc)] x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells.

Hepatoprotective Percent = % Viability of treatment group – % Viability of negative control.

DETERMINATION OF *IN-VIVO* HEPATOPROTECTIVE ACTIVITY

Experimental animals

Male Wistar albino rats (100 ± 2 g) were obtained from the Animal House, National Research Center (NRC), Giza, Egypt. They were housed in plastic cages with a room temperature of 22 ± 1°C under a 12 h light-dark cycle and fed a standard diet of commercial rat chow, tap water ad libitum. The animals were allowed one week under these conditions to acclimatize before the commencement of the experiment. The studies were carried out in accordance with the Institutional Ethics Committee and in accordance with the recommendations for the proper care and use of laboratory animals (Committee number 8, 19-11-2015, code 21).

Determination of median lethal dose (LD_{50})

The LD_{50} of compounds 5& 6 was estimated according to Kärber's procedure. The animals were divided into groups, each of six animals. Preliminary experiments were carried out to determine the minimal dose that kills all animals (LD_{100}) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were chosen in between these two doses; each dose was injected in a group of six animals. The number of dead animals in each group, 24 hours after injection was determined and the median lethal dose (LD_{50}) was calculated.

Experimental design

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 15 days followed by administration of CCl4 (1.5 mg/kg b.wt., interpritonial) twice weekly until day 21.

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., interpritonial) twice weekly until day 21.

Group VI: Rats were orally administered 70% dried methanol extract of *Agathis robusta* solubilized in distilled water (100 mg/kg b.wt.) for 15 days followed by administration of CCl4 (1.5 mg/kg b.wt., interpritonial) twice weekly until day 21.

Group VII: Rats were orally administered compound 5 solubilized in distilled water (1 mg/kg b.wt.) for 15 days followed by administration of CCl4 (1.5 mg/kg b.wt., interpritonial) 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., interpritonial) twice weekly until day 21.

Biochemical assessment

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.[14]

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]

Compound 1 (C1): is identified as; 3-O-[α-D-glucopyranosyl-(1→4) - α -L-rhamnopyranosyl-(1→2)]- α -L-arabinopyranosyloleanolic acid.[15,16]

Compound 2 (C2): 3 -O- {[α-D-glucopyranosyl-(1→3) - α -L-rhamnopyranosyl-(1→2)] - α -D-glucopyranosyl-(1→4)} - α -L-arabinopyranosyloleanolic acid.[15]

Compound 3 (C3): 3, 23-dihydroxy-12-oleanen-28-oic acid, 3-O-[α-L-rhamnopyrosyl-(1→2)-α-L-arabinopyranoside]-28-O- [α-L-rhamnopyrosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside].[17]

Compound 4 (C4): 3, 23-dihydroxy-12-oleanen-28-oic acid, 3-O-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside]-28-O-[α-D-glucopyranoside]. [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 $C_{53}H_{86}O_{22}$. 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-162-146-162]$^-$ 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$^-$ = $[C_{30}H_{48}O_4\text{-}H]^-$ = $[C_{30}H_{47}O_4]^-$) 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 arabino pyranosyl moiety.[17,19]

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 HMQC 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 ^{13}C-NMR were seen in association with the signals at 122.76 (CH-12) and 144.95 (C-13).

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

Sample	Hepatoprotective effect (EC_{50}) µg/ml
C5	< 5000
C6	< 5000
Silymarin (Standard drug)	150

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

Group No.	ALT	AST
Group I	11.13±1.12	22.60 ± 2.21
Group II	60.11±2.1	92.73 ± 2.7
Group III	40.1 ± 3.3	46.1 ±2.2
Group IV	48.1 ± 3.1	45.7 ± 2.9
Group V	49.07 ± 3.93	28. 4± 1.5
Group VI	30.6 ± 2.03	28.44± 1.55
Group VII	31.9 ± 2.2	27.87± 2.25
Group VIII	30.9 ± 3.2	28.44± 1.58

Values were expressed as mean ± standard deviation in each group.

As an aglycone, NMR spectra revealed a pentacyclic triterpenoid skeleton from olean-12-ene. The cross peaks detected in the HMQC 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 ^{1}H and ^{13}C.

The [-D-galactopyranosyl-(1→3)-L-rhamnopyranosyl-(1→2)], β-D-glucopyranosyl-(1→4)-a-L-arabinopyranosyl moiety was primarily characterised and located at CH-3 using the HMBC spectrum, which displayed cross peaks matching to CH-3.

The structure of the current triterpenoid glycoside (C5) has therefore been described as 3-O-{[β-D-galactopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)] - β-D-galactopyranosyl-(1→4)}-α-L-arabinopyranosyl hederagenin.

Compound 6 (C6) was obtained as buff amorphous powder. CH_2OH group [δC 63.1; H 4.30 (d, J= 11.7 Hz) and 3.89 (d, J= 11.7 Hz)] was changed by a methyl group (δH 1.39 and δC 29.64) in C6. The ^{1}H and ^{13}C spectrum data of the saponin (C6) were compared to those of C5. The ^{13}C chemical changes of the CH-3 at δC 90.02 (δC 81.47 in C5), CH-5 at δC 57.37 (δC 48.31 in C5) and CH_3-24 at δC 18.43 (δC 14.26 in C5) all verified this replacement.

The oligosaccharide moiety,[17-19] in the triterpenoid C5 with the same kind and sequence of sugars chain with one additional glucopyranosyl group in saponin 6, explained significant additional discrepancies seen in the comparative examination of the NMR spectra of C5 and C6.

MS showed a molecular ion peak at m/z 1220 ([M]$^-$) compatible with a molecular formula $C_{59}H_{96}O_{26}$. Other significant negative ion peaks were observed at m/z 1057 ([M-163]$^-$, 895 ([M-163-162]$^-$, 749 ([M-163-162-146]$^-$, 733 ([M-163-162-162]$^-$, 587 ([M-163-162-162-146]$^-$, and 455 ([M-163-162-162-146-132]$^-$).

The values corresponding to vicinal spin-spin interaction (3JH, H) between the anomeric hydrogens of arabinopyranosyl [J= 7.5 (C5) and 6.7 Hz (C6)] and galactopyranosyl (J= 7.8 to 7.9 Hz) moieties are consistent with axial-axial couplings and, consequently, the configuration of the anomeric carbons was defined as β for galactose and α for arabinose.[18] Thus, the structure of compound 6 is 3-O-{[β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)]-[β-D-galactpyranosyl-(1→4)]}-α-L-arabinopyranosyloleanolic acid.

Table 1 showed the strong hepatoprotective activity of compound 5 (C5) and compound 6 (C6) on the tested hepatic cells (HepG-2).

Table 2 shows the results of the hepatoprotective effects of C5 and C6 on ALT and AST activities. When administered with a middle concentration of both C5&C6 (1 mg/kg b.wt), both ALT and AST activity dropped significantly (p 0.05) when compared to the model group.

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.

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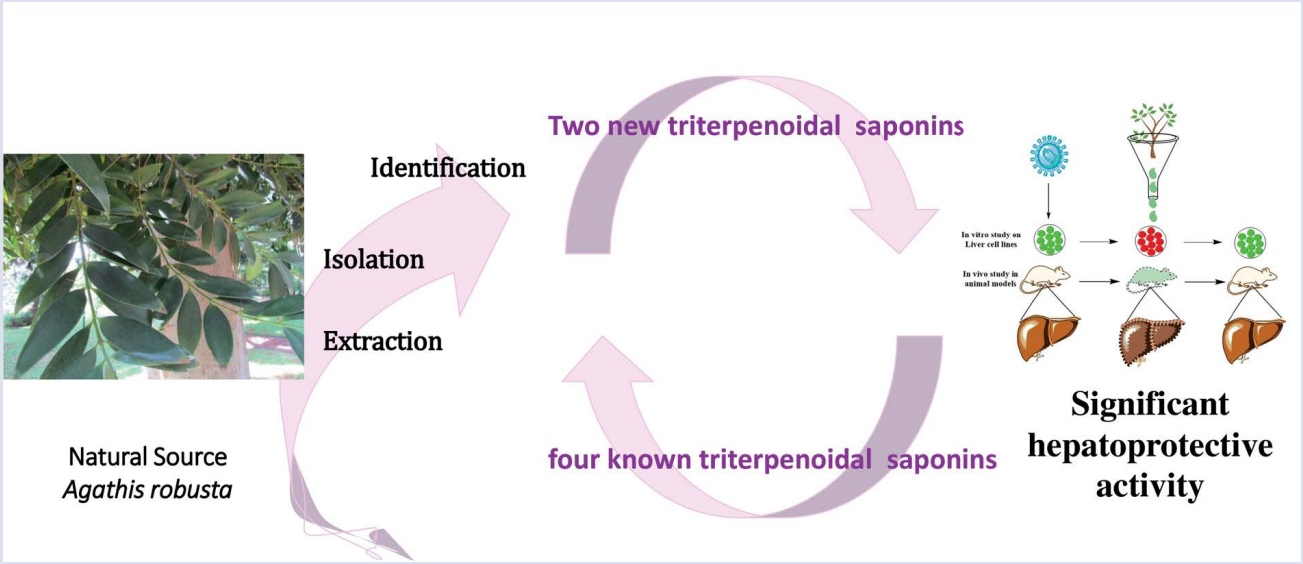
GRAPHICAL ABSTRACT



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