INTRODUCTION
Cancer affects millions of people worldwide despite of the improved molecular diagnostic techniques [1]. Accordingly, cancer is a clinically serious problem which possesses significant social and economic changes to the health care system. Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third public reason of cancer linked death throughout world [2]. The synchronous existence of HCC strength may be due to numerous hazard factors such as chronic viral infection diseases with hepatitis B virus (HBV) and hepatitis C virus (HCV), aflatoxin exposure, alcohol drinking, drugs consumption or iron overload [3, 4]. In maximum cases, the recovery proportion from HCC is short and existing predictable and adapted therapies are hardly beneficial [5, 6]. Thus, there is an urgent need for new therapeutic agents for HCC patients. A collective means of drug finding is the ethno-medical method, in which the choice of a plant is found on its usage as folkloric system. A large number of anti-cancer drugs have been extracted from plants containing phenolic compounds as flavonoids, tannins, steroid and terpenoids, etc. [7-9]. Plants containing polyphenols, flavonoids and/or tannins received considerable attention in recent years for their biological activities [10-14]. For example some species of *Afrocarpus* genus (family Podocarpaceae) reported to have several biological activities such as antiradical, anti-inflammatory, anti-viral, cytotoxic, anti-microbial properties. These biological activities were revealed for their contents of terpenoid, tannins and flavonoids such as Apigenin 8-C-β-D-gluco-pyranosyl-(1''→4'')-O-β-D-glucopyranoside, Queiretin 3-O-β-D-glucopyranoside and II-4'',1-7-dimethoxyamentoflavone [15-20]. *Afrocarpus gracilior* (syn. *Podocarpus gracilior*) is an interesting species growing in Egypt which has documented as anti-oxidant and identified to contain taxol [18, 20-22]. Therefore, the aim of this study was to take an overview and to continue isolating the potential components responsible for the cytotoxic activity from *Afrocarpus gracilior*. 

**Fig. 1:** Chemical structures of two new flavones (1, 2) and standard drug doxorubicin

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ANTICANCER ACTIVITY AGAINST HEPATOCELLULAR CARCINOMA

**ABSTRACT**

Objective: Cancer is considered as one of the top reasons of death and the number of cases increasing gradually. Cancer is severe clinical difficulty to the health caution system. This study explored two novel polyphenols of *Afrocarpus gracilior* Pliger growing in Egypt and evaluated their cytotoxic activity.

Methods: Methanolic (80%) extract of the leaves of *A. gracilior* was subjected to column chromatography; the chemical structures of the isolated compounds were established by advanced spectral techniques: UV, 1H, 13C NMR, two dimensional NMR (2D NMR) and electron spray ionization mass spectroscopy (ESI-MS). Compounds 1 and 2 were studied for their cytotoxic activity against hepatocellular carcinoma (Hep-G2) using sulforhodamine B (SRB) assay. Furthermore the pharmacokinetics profiles of these molecules were accessed by employing Petra/Orisiris/Molinspiration (POM) analyses.

Results: Two novel C-flavonoid glycosides were isolated [1: Apigenin 8-C-β-D-gluco-pyranosyl-(1''→4'')-O-β-D-glucopyranoside] and [2: 7-O-methyl-luteolin 8-C-β-glucopyranosyl(1''→4'')-O-β-D-gluco-pyranoside]. They exhibited significant cytotoxic activity (IC₅₀ = 9.02 and 15.61 µg/ml respectively) against Hep-G2 cells. The POM analyses revealed that the activity of these two compounds depends on the presence of glucosyl and alkyl groups at the internal and terminal atmosphere of the compounds.

Conclusion: These findings demonstrated that the leaves of *A. gracilior* contain a series of bioactive polyphenolic compounds with significant cytotoxic properties against hepatocellular carcinoma and may be used as alternative anticancer agents for doxorubicin. On the basis of POM calculations, it will be interesting to develop some alternative flavones because the deglucosylated derivatives have a better drug score than parent molecules. This preliminary study will be extended to other strains of cancer.

**Keywords:** Podocarpaceae, *Afrocarpus*, flavonoid glycosides, Anticancer activity, Petra/Orisiris/Molinspiration (POM) analyses

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MATERIALS AND METHODS

Apparatus

\(^1H\) and \(^13C\) NMR spectra were obtained on a Bruker AX500 at 500 and 125 MHz, respectively. The mass spectra (MS) were verified on a Waters Acquity Ultra Performance LC with ZQ detector in ESI mode. The UV studies for compounds (1 and 2) were measured on a Shimadzu UV-240 spectrophotometer, distinctly as solutions in methanol and with diagnostic UV shift reagents [23, 24]. Rotary evaporator (Büchi, G, Switzerland) was used for evaporation.

Plant material

Identification of Afrocarpus gracilior Pilger (syn. Podocarpus gracilior) confirmed by Dr. Terase Labib, El-Orman Garden, Cairo, Egypt. Voucher specimen (Reg. no. 02Pgr/2018) was kept in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

Spectroscopic data of compounds 1 and 2

**Compound 1:**

Pale yellow amorphous powder (10 mg). Chromatographic properties: Rf values; 0.29 (S1) and 0.28 (S2) as shown in Table 1. Ultraviolett l/2 (VL-215 LC, Marne La Vallee, France) was used for visualization of compounds spots on paper chromatograms and follow up various column fractions on columns at 254 or 365 nm and also with located (sprayed) Narturstoff and FeCl$_3$ reagents [25].

**Plant material**

**Compounds 1 and 2**

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**RESULTS AND DISCUSSION**

Investigation of polyphenolic contents (Extraction and isolation)

Powdered, air-dried leaves of *A. gracilior* (1050 g) were exhaustively extracted with hot 80% MeOH (5×3 l) under reflux. The dry residue obtained (140 g) was extracted with chloroform (3×1 l). The 2D-PC revealed that chloroform soluble portion contains limited polyphenolic contents, while they were concentrated in methanol soluble portion. The aqueous residue (120 g) was fractionated on a polyamide column (300 g. 0.5×120 cm) using a step gradient H$_2$O/MeOH mixture with decreasing polarity from 100% water to MeOH 100% for elution to yield 25 individual fractions, collected chromatographically two pure compounds (1 and 2) identified on the basis of acid hydrolysis, comparative PC, UV, ESI-MS, \(^1H\), \(^13C\)-NMR and 2D-NMR spectroscopic analyses (fig. 2-3).
Structure elucidation of 1, 2

The dried residue of 80% MeOH extract, which was extracted with chloroform for defatting and aglycone extraction [28] was chromatographed on a polyamide column followed by successive separation on sephadex LH-20 and cellulose columns affording two pure compounds, among which was compound 1, that exhibited chromatographic properties, UV-spectral data of C-glycosylapigenin. The UV spectrum in MeOH exhibited the two characteristic absorption bands at λmax (nm) 272 nm (band II) and 335 nm (Band I) of apigenin nucleus. On addition of NaOH, bathochromic shift of band II (47) was diagnostic for free 7-OH group. The remaining diagnostic shift reagents were in complete accordance with 5, 7, 4'-trihydroxy-C-glycosyl flavones structure [24]. Negative ESIMS spectrum exhibited the molecular ion peak at m/z 593 [M-H]− corresponding to M. wt. of 594, molecular formula C27H30O16 [34] and fragment ion peak at m/z 451 after loss of a glycosyl moiety indicating apigenin dihexoside structure. 1H-NMR spectrum showed an AX coupling system of two ortho doublets, each integrated for two protons at δ ppm 8.12 and 7.06 assigned to H2 /6′ and H3-3′/5′, respectively of 1′, 4′-disubstituted ring-B, in addition to the two singlet signal resonances at δ ppm 6.64 and 6.46 assignable to H-3 and H-4, respectively characteristic for an apigenin moiety missing H-8 resonance signal. The two anemic protons appeared as doublets at δ ppm 5.06 with large J value 9.9 Hz and 4.74 with J value 7.4 Hz, gave the suggestion of presence of a C-gluco-side and O-gluco-side moieties with a β-linkage, respectively. The absence of H-8 gave the expectation of C-glucosidation on C-8. This signal was established from downfield shift of 13C-resonance of C-8 to δ ppm 104.59 [α+10 ppm] in 13CNMR spectrum. Moreover, the C-glucoside moiety was confirmed as β-glucopyranoside depending on the characteristic upfield location of C-1′ at δ ppm 72.04 and downfield locations of C-5′ and C-3′ at δ ppm 82.69 and 79.59, respectively with respect to those of O-gluco-side. The presence of another six carbon resonances with the anomeric carbon at δ ppm 101.25 characteristic for β-O-glycopyranoside structure confirming the presence of a second glucose moiety. In addition, the downfield shift of C-4′ at δ ppm 77.57 was an evidence for 1′-4′ intra glycosidic linkage. HMBC approved the linkage between the two glucosyl moiety glucopyranosyl-(1′→4′) of β-O-glycopyranoside (fig. 2). All 1H and 13C resonances were assigned by comparison with the corresponding values of structurally related compounds of previously published data [20, 29-33]. In the light of these data compound 1 was identified as Apigenin 8-C-β-D-glucopyranosyl-(1′→4′)-O-β-D-glucopyranoside (Vitexin 4′-O-β-D-glucopyranoside) which is isolated for the first time from nature (fig. 2).

According to the chromatographic properties, compound 2 was expected to be a glycosyl luteolin [34]. UV-spectrum in MeOH displayed the two distinctive absorption bands I and II of luteolin nucleus at λmax 350 and 258 nm, respectively. On addition of NaOH, no bathochromic shift of band II was observed which is diagnostic for a substituted 7-OH group. The bathochromic shift of band I in ACl1 together with hypochromic shift experimental after adding of HCl confirmed the occurrence of ortho-dihydroxy groups at C-3′ and C-4′ in ring B, still the bathochromic shift in band II relative to MeOH continued after adding of HCl designated the occurrence of a free 5-OH group. Confirmation of the given the chromatographic properties, compound 2 was expected to be 5,3′,4′-trihydroxy glycosyl flavone [34, 35]. Negative ESIMS spectrum exhibited the molecular ion peak at m/z 623 [M-H]−, corresponding to molecular weight of 624 and molecular formula C30H28O14, to support evidence of methyl luteolin-di-hexoside structure. In 1H NMR spectrum a flavone compound was confirmed by the appearance of a singlet at δ 6.62 for H-3. Additionally, the spectrum showed an ABX-spin coupling system of three proton resonances at 7.91 (H-2), 7.85 (H-6), and ortho doublet at 6.92 (H-5) to indicate a 3′, 4′-dihydroxy B ring indicating a luteolin nucleus. The absence of H-8 signal from the spectrum and the presence of doublets of large J-values 9.7 Hz at 4.99 ppm was attributed to anomeric proton of C-β-D-glucopyranoside moiety. Another anomeric proton appeared as doublets at δ ppm 4.7 with J value 7.8 Hz, gave the suggestion of presence of O-glucoside moiety with a β-linkage. As further confirmation, 13C NMR spectrum showed well-resolved typical 15 signals of a luteolin aglycone moiety, including the three key signals of C-3′, C-4′ and C-3 at δ ppm 146.60, 150.55 and 102.77, respectively. The downfield shift of 13C resonance of C-8 to δ 105.77 was confirmative evidence for the C-glucosidation at C-8. Moreover the C-glucoside moiety was confirmed as 8-C-β-D-glucopyranoside depending on the intrinsic upfield location of C-1′ (anomeric carbon) at δ 71.25 ppm. The presence of another six carbon resonances with the anomeric carbon at δ ppm 101.25 characteristic for β-O-glycopyranoside structure confirming the presence of a second glucose moiety. HMBC approved the linkage between the two glucosyl moiety glucopyranosyl-(1′→4′) of β-D-glucopyranoside (fig. 2). The presence of carbon resonance at δ 55.98 together with The upfield shift of C-7 at 162.57 ppm were indicative for the presence of methoxy group attached to C-7 which was approved by HMBC spectrum (fig. 3). This structure is confirmed by comparison with previous published reports [34-37]. Hence, compound 2 was identified as 7-0-methyl-luteolin 8-C-β-glucopyranosyl-(1′→4′)-O-β-D-glucopyranoside (Vitexin 4′-O-β-D-glucopyranoside) which is isolated for the first time from nature (fig. 3).

Evaluation of cytotoxic activity of 1, 2

Compounds 1 and 2 showed significant cytotoxic activities against Hep-G2 hepatocellular carcinoma (IC50 values 902±0.54 and 15.61±0.23 μg/ml, respectively) compared to doxorubicin as a standard drug (IC50 value 4.47±0.13 μg/ml) as shown in table 2 and fig. 4-5.

Table 2: Comparative bioactivity of compounds 1, 2 and doxorubicin as standard drug

| Bioactivity of compound 1 | Bioactivity of compound 2 | Bioactivity of doxorubicin |
|--------------------------|--------------------------|--------------------------|
| Conc. (μg/ml) | Average | SD | Conc. (μg/ml) | Average | SD | Conc. (μg/ml) | Average | SD |
| 5 | 0.620333 | 0.014012 | 5 | 0.667667 | 0.01365 | 0.5 | 0.75 | 0.004583 |
| 10 | 0.468667 | 0.020207 | 10 | 0.538667 | 0.017214 | 1 | 0.637 | 0.007937 |
| 25 | 0.333333 | 0.007767 | 25 | 0.436 | 0.014 | 2.5 | 0.571 | 0.004 |
| 50 | 0.194333 | 0.005217 | 50 | 0.306667 | 0.01066 | 5 | 0.481333 | 0.005508 |
| 100 | 0.127 | 0.009165 | 100 | 0.214333 | 0.010599 | 10 | 0.306667 | 0.010066 |

Antitumor activity of 1, 2

The effect of both compounds 1 and 2 were tested against Hep-G2 using the SRB method. SRB dye was used as a stain to estimate cell number indirectly [27]. The National Cancer Institute (NCI) indicated that the cytotoxicity of a plant extract is considered effective with the IC50 below 20 μg/ml [38]. Compounds 1 and 2 isolated from P. gracilis have significant cytotoxic activity against...
Hep-G2 with IC\textsubscript{50} values 9.02 and 15.61 µg/ml, respectively (fig. 4 and 5). Accordingly compounds 1 and 2 could be used as chemopreventive agents since recent studies suggested that using plant derived chemopreventive agents in combination with chemotherapy can increase the usefulness of chemotherapeutic agents and lessen their toxicity to normal tissues [39, 40].

Fig. 4: Cytotoxic activity of compound 1 against Hep-G2 cell line (n = 3)

Fig. 5: Cytotoxic activity of compound 2 against Hep-G2 cell line (n = 3)

**POM analyses of compounds 1, 2**

A potential drug should not have only a good bioactivity, it must have acceptable pharmacokinetic properties. To access the pharmacokinetic profile of molecules, we employed Osiris, Petra and Molinspiration (POM) as a good virtual screening with about 7000 drugs available on the market.

POM analyses of the standard drug, molecules 1, 2 and their deglucosylated derivatives (1', 2', 2'') revealed that derivatives of 1, 2, contrary to reference drug and the two new flavones 1, 2 are more active (tables 3, 4). They showed better drug scores and can be utilized as therapeutic agents. In fact, structures of the investigated anti-cancer drugs are supposed to present some risks when run through the mutagenicity, tumorigenicity assessments, and that these two compounds were at low risk comparable with standard drug (SD) as near as irritation and reproductive effects are concerned.

For example, acute side-effects of doxorubicin include vomiting, nausea, and heart arrhythmias. It can also causes neutropenia (reduction in white blood cells) and alopecia (hair loss). An increasing dose of doxorubicin is capable to lead the patient to severe risks of developing cardiac side effects; including congestive heart failure, dilated cardiomyopathy, and death, powerfully rise. Reactive oxygen species (ROS), formed by the contact of doxorubicin with iron, can then harm the myocytes [heart cells], creating myofibrillar loss and cytoplasmic vacuolization [41].

The hydrophilicity character of each constituent had been stated in terms its cLog\textsubscript{P} value since it had been recognized that the absorption or permeation is importantly affected by this quantity (value of cLog\textsubscript{P}). Therefore, when the value of cLog\textsubscript{P} is greater than 5, the absorption or permeation decreases. Our results showed that the new compounds (1 and 2) have similar cLog\textsubscript{P} of those of the anti-tumor standard drug SD, within the acceptable criteria. As the molecular weight of all parent molecules (1, 2 and SD) is 594-624 g, it is necessary to realize more chemical modification (deglycosylation) in goal to make more potentially active analogues.

The actual drug-scores of compounds 1 and 2 are very encouraging (positive value of DS) as shown in table 4.

**Table 3: Molinspiration calculations of compounds 1, 2 and doxorubicin**

| Compound | Molecular properties a | Bioactivity scores b |
|----------|------------------------|----------------------|
|          | TPSA | ONH | VIOL | VOL | GPCR | ICM | KI | NRL | PI | EI |
| 1        | 260  | 10  | 3    | 487 | 0.10 | -0.45 | -0.01 | -1.04 | 0.06 | -0.28 |
| 2        | 269  | 10  | 3    | 513 | 0.01 | -0.68 | -0.16 | -1.32 | -0.04 | 0.11 |
| SD b     | 209  | 8   | 3    | 465 | 0.27 | -0.14 | 0.08  | 0.24  | 0.56  | 0.64 |

\(\text{TPSA: Total of Polar surface area; ONH: O—H—N or O—HN Interaction; VOL: Volume,}\) \(\text{GPCR: GPCR ligand; ICM: Ion channel modulator; KI: Kinase inhibitor; NRL: Nuclear receptor ligand; PI: Protease inhibitor; EI: Enzyme inhibitor.}\) \(\text{Standard drug: (SD = Doxorubicin). Structure of SD is given in fig. 1.}\)
### Table 4: Molinspiration prediction of compounds 1, 2 and doxorubicin and their hypothetic derivatives (1’, 2’, 2”, SD’ and SD")

| Compound | Structure | Molecular Properties | Drug Scores |
|----------|-----------|----------------------|-------------|
| 1        | ![](chart1.png) | miLogP: -1.41 | GPCR ligand: 0.10 |
|          |           | TPSA: 260         | Ion channel modulator: -0.45 |
|          |           | MW: 594           | Kinase inhibitor: -0.01 |
|          |           | nON: 15           | Nuclear receptor ligand: -0.04 |
|          |           | nOHNH: 10         | Protease inhibitor: 0.06 |
|          |           | nviolations: 3    | Enzyme inhibitor: 0.28 |
|          |           | volume: 487       |             |
| 1’       | ![](chart2.png) | miLogP: 0.52      | GPCR ligand: 0.13 |
|          |           | TPSA: 181         | Ion channel modulator: -0.14 |
|          |           | MW: 432           | Kinase inhibitor: 0.19 |
|          |           | nON: 10           | Nuclear receptor ligand: 0.23 |
|          |           | nOHNH: 7          | Protease inhibitor: 0.03 |
|          |           | nviolations: 1    | Enzyme inhibitor: 0.46 |
|          |           | volume: 355       |             |
| 2        | ![](chart3.png) | miLogP: -1.61     | GPCR ligand: 0.01 |
|          |           | TPSA: 269         | Ion channel modulator: -0.68 |
|          |           | MW: 625           | Kinase inhibitor: -0.16 |
|          |           | nON: 16           | Nuclear receptor ligand: -0.32 |
|          |           | nOHNH: 10         | Protease inhibitor: -0.04 |
|          |           | nviolations: 3    | Enzyme inhibitor: 0.11 |
|          |           | volume: 513       |             |
| 2’       | ![](chart4.png) | miLogP: 0.10      | GPCR ligand: 0.10 |
|          |           | TPSA: 190         | Ion channel modulator: -0.17 |
|          |           | MW: 462           | Kinase inhibitor: 0.16 |
|          |           | nON: 11           | Nuclear receptor ligand: 0.16 |
|          |           | nOHNH: 7          | Protease inhibitor: -0.04 |
|          |           | nviolations: 2    | Enzyme inhibitor: 0.40 |
|          |           | volume: 381       |             |
| 2”       | ![](chart5.png) | miLogP: 0.03      | GPCR ligand: 0.12 |
|          |           | TPSA: 201.27      | Ion channel modulator: -0.14 |
|          |           | MW: 448.38        | Kinase inhibitor: 0.19 |
|          |           | nON: 11           | Nuclear receptor ligand: 0.20 |
|          |           | nOHNH: 8          | Protease inhibitor: 0.01 |
|          |           | nviolations: 2    | Enzyme inhibitor: 0.45 |
|          |           | volume: 363.22    |             |
| SD       | ![](chart6.png) | miLogP: 0.07      | GPCR ligand: 0.27 |
|          |           | TPSA: 209.24      | Ion channel modulator: -0.14 |
|          |           | MW: 545.54        | Kinase inhibitor: 0.08 |
|          |           | nON: 12           | Nuclear receptor ligand: 0.24 |
|          |           | nOHNH: 8          | Protease inhibitor: 0.56 |
|          |           | nviolations: 3    | Enzyme inhibitor: 0.64 |
|          |           | volume: 465.04    |             |
| SD’      | ![](chart7.png) | miLogP: 0.65      | GPCR ligand: 0.25 |
|          |           | TPSA: 165         | Ion channel modulator: 0.04 |
|          |           | MW: 416           | Kinase inhibitor: -0.08 |
|          |           | nON: 9            | Nuclear receptor ligand: 0.39 |
|          |           | nOHNH: 6          | Protease inhibitor: 0.20 |
|          |           | nviolations: 1    | Enzyme inhibitor: 0.45 |
|          |           | volume: 346       |             |
| SD”      | ![](chart8.png) | miLogP: 0.37      | GPCR ligand: 0.28 |
|          |           | TPSA: 176         | Ion channel modulator: 0.09 |
|          |           | MW: 402           | Kinase inhibitor: -0.06 |
|          |           | nON: 9            | Nuclear receptor ligand: 0.45 |
|          |           | nOHNH: 7          | Protease inhibitor: 0.26 |
|          |           | nviolations: 1    | Enzyme inhibitor: 0.53 |
|          |           | volume: 328       |             |

### CONCLUSION

The methanolic extract of the leaves of *A. gracilior* contains a considerable amount of polyphenolic compounds that have significant cytotoxic properties and thus have great potential as a source for natural health products. The POM analyses revealed that the activity of compounds 1, 2 and standard drug (SD) depended on the presence of glucosyl and alkyl groups at the internal and terminal atmosphere of the compounds. The docking analysis revealed that lipophilic and H-bonding interactions were the prominent interactions among flavones and the Cancer-DNA receptor. The POM Analyses of compounds 1, 2 and SD proved to be a useful tool in the prediction of anti-tumor activity of congeneric compounds and some important insights were also originate that will be useful to monitor for the prediction of new cancer inhibitors with enhanced bio-activity.
AUTHORS CONTRIBUTIONS

AMK, MISA, involved in selection, collection of the plant, extraction and isolation. AMK contributed in structural elucidation and MISA in biological analysis. AMK, MISA involved in writing the manuscript. THB contributed in spectral and POM analyses. All authors have read the final manuscript and approved the submission.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest

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