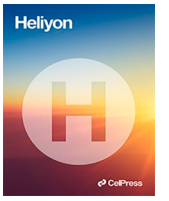
Heliyon 9 (2023) e20034

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Phytochemical and metabolic profiling of the different *Podocarpus* species in Egypt: Potential antimicrobial and antiproliferative activities

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ARTICLE INFO

Keywords:
Podocarpaceae
Taxol
Metabolomic profiling
ITS region
GC/MS profile
Bioactivity

ABSTRACT

Podocarpus is the most dominant genus of Podocarpaceae, with higher taxonomical proximity to the Taxaceae, having numerous pharmaceutical applications, however, scarce studies dealing with the physiological and metabolic criteria of *Podocarpus* in Egypt were reported. Thus, the objective of this work was to assess the physiological and metabolical patterns of the different species of *Podocarpus*; *P. gracilior, P. elongates, P. macrophyllus* and *P. neriifolius.* The highest terpenoids contents were reported in *P. neriifolius*, followed by *P. elongatus*, and *P. macrophyllus*. *P. gracilior* had the highest antioxidants amount, followed by *P. macrophyllus*, *P. neriifolius* and *P. elongatus*. From the GC/MS metabolic profiling, caryophyllene, β-cadinene, β-cuvebene, vitispirane, β-cadinene and amorphene were the most dominant metabolites in *P. gracilior*. β-Caryophyllene was the common in *P. gracilior, P. elongatus, P. macrophyllus* and *P. neriifolius* with an obvious fluctuation. The plant methanolic extracts have an obvious activity against the multidrug resistant bacteria; *E. coli*, *P. aeruginosa*, *S. pyogenes* and *S. aureus*, and fungi; *A. fumigatus*, *A. flavus, A. niger* and *C. albicans* in a concentration-dependent manner. The highest Taxol yield was assessed in the extracts of *P. elongatus* (16.4 μg/gdw), followed by *P. macrophyllus*, and *P. neriifolius*. The chemical identity of Taxol derived from *P. elongatus* was resolved by LC/MS, with molecular mass 854.6 *m/z*, and similar structural fragmentation pattern of the authentic one. The highest antitumor activity of *P. elongatus* extracted Taxol was assessed towards HCT-116 (30.2 μg/ml), HepG-2 (53.7 μg/ml) and MCF-7 (71.8 μg/ml). The ITS sequence of *P. elongatus* "as potent Taxol producer" was deposited on Genbank with accession #ON540734.1, that is the first record of *Podocarpus* species on Genbank.

1. Introduction

Medicinal plants are the frequent traditional natural source of diverse bioactive secondary metabolites especially terpenoids, flavonoids, alkaloids, saponins, tannins, sterols, and antioxidants. Among the medicinal plants, Podocarpaceae is one of the most recognized plant families with various valuable therapeutic activities such as anti-inflammatory, anticancer, antimicrobial,

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https://doi.org/10.1016/j.heliyon.2023.e20034
Received 21 October 2022; Received in revised form 4 August 2023; Accepted 8 September 2023
Available online 9 September 2023

antioxidant and antidiuretic [1,2]. The water extract of the red seaweed *Kappaphycus alvarezii* displayed a powerful antioxidant, antibacterial activity against various pathogenic bacteria [3]. The medicinal plants *Centella asiatica* and *Allium sativum* have been used as source of bioactive compounds against various pathogenic microbes such as *Candida albicans*, *Aspergillus niger* and *Penicillium* sp [4,5]. Podocarpaceae (Yellow Wood) is one of the largest coniferous family after Pinaceae [6], including seven genera namely *Podocarpus*, *Dacrydium*, *Phyllocladus*, *Acmopyle*, *Microcachrys*, and *Saxegothaea*. The genus *Podocarpus* is usually with evergreen shrub or tree habit, dioecious, simple linear-lanceolate or linear-elliptic foliage with alternate, opposite or spiral arrangement, solitary ovulate cones with swollen, and fleshy seeds [7,8]. *Podocarpus elongatus* has been morphologically characterized with spirally arranged, narrowly elliptic, light green-blue colored entire simple leaves. *Podocarpus macrophyllus* (Thunb.) has a broad leaf with congested and spirally arranged strap-shaped entire simple leaves, dark green on top, grayish underneath, and tapered at the ends. *Podocarpus neriifolius* has a spiral arranged leaves, slightly curved lanceolate deep green simple broad leaves, and obtuse at tips [6].

The genera of Podocarpaceae have been implemented in various technological applications including dyes, waxes and tanning leather manufacturing [1]. *Nageia nagi* has been used as herbal dietary supplement, fleshy receptacle fruits of *P. dacrydioides, P. nivalis, P.totara,* and *P. salignus* have been eaten [1]. Young leaves and stems of *P. nagi* are eaten [9], their seeds were used in oil industry [10]. Species of Podocarpaceae have been used as an herbal remedy in traditional medicine for treatment of various human and animal diseases [11]. The stem and bark extracts of *P. macrophyllus* are used in treatment of blood disorders and ringworms [12]. A decoction of *P. neriifolius* leaves has been used in treatment of painful joints and rheumatism [13]. Overall, the extracts of *Podocarpus* spp are used in treatment of venereal diseases, fevers, asthma, coughs, cholera, distemper and chest complaints [1]. From the phytochemical profiles, myriad of bioactive chemical constituents were identified from the leaves and receptacles of *Podocarpus* spp, especially diterpenoids, flavonoids and antioxidants with useful cytotoxic properties and biological activities [11,14]. Among these bioactive compounds, Taxol is one of the most successful anticancer diterpenoid that was isolated from stems of *P. gracilior* [15], consequently, use of the medicinal plant extracts were reported to be an alternative to the traditional antibiotics [16]. The current study was an extension to our previous work [17–19], to assess the metabolic traits especially Taxol contents of the different *Podocarpus* species inhabiting Egyptian botanical gardens. With the myriads of predicted bioactive compounds from *Podocarpus* spp, these plants receive less attention in Egypt. Thus, the objective of this work was to explore the physiological and metabolic traits of different *Podocarpus* species, to assess their potential antimicrobial activity against the multidrug resistant microorganisms, extraction of Taxol and evaluating their anticancer activity.

2. Materials and methods

2.1. Chemicals

Authentic Taxol (Cat. #T7402), linalool (Cat. #L2602), quercetin (Cat. #Q3001), gallic acid (Cat. #G7384), L-ascorbic acid (Cat. #A92902) and bovine serum albumin (Cat. # A9056) were obtained from Sigma-Aldrich (MO, USA). All other chemicals and reagents were of analytical grades.

2.2. Collection of the Podocarpus samples

Four species of the genus *Podocarpus* inhabiting Egyptian botanical gardens were collected from different localities in Egypt in June/2021. Fresh and healthy leaves sample of *Podocarpus gracilior* (Pilg.) and *P. elongatus* (Aiton) L'Hér. ex Pers., were collected from Botanic Garden of the Faculty of Science, Alexandria University and Al-Zohriya garden, Giza, Egypt, respectively. The leaves of *P. macrophyllus* (Thunb.) D. and *P. neriifolius* D. were obtained from Aswan Botanical Garden, Aswan, Egypt. The plants were identified according to their taxonomic characteristic features [20–30].

2.3. Phytochemical analyses

2.3.1. Preparation of methanolic crude extracts

Fresh leaves of the experimented *Podocarpus* spp were washed, air-dried indoors at room temperature on sterile paper for 20 days. After drying, the plant samples were individually pulverized into fine powder, then stored in sterile vials to avoid microbial contamination, till used for extraction. Methanolic extracts of the leaves were prepared [31], with slight modifications. Briefly, 10 g of the air-dried powdered leaves of each *Podocarpus* species were cold macerated by soaking in 80 ml of absolute methanol with occasional shaking at 30 °C for 24 h, using a rotary shaker. The crude methanolic extracts were filtered by sterile cheesecloth, centrifuged at 5000 g for 15 min, and the supernatant was collected. The extracts from the three consecutive extractions were combined and concentrated by rotary evaporator at 40 °C, and the obtained oily residues were suspended in 10 ml absolute methanol. The extraction yield of methanolic crude extracts was quantified [32,33] according to the following equation:

$$\text{Extract yield } (\%)\ (\text{W}/\text{W}) = \frac{\text{Weight o f extract } (\text{g}) \text{ after solvent evaporation}}{\text{Weight o f the dried plant sample before extraction } (\text{g})} \times 100 \quad (1)$$

The crude methanolic extracts were stored at −20 °C, for the ongoing analysis.

2.3.2. Phytochemical analyses

The total alkaloids, flavonoids, phenolic compounds, carbohydrate and proteins were estimated as follow.

2.3.3. Qualitative analyses

The methanolic extracts of the leaves of *P. gracilior*, *P. elongatus*, *P. macrophyllus* and *P. neriifolius* were analyzed to identify their active constituents; terpenoids, phytosterols, flavonoids, alkaloids, saponins, tannins, phlobatannins, glycosides, anthraquinone glycosides, anthraquinone, carbohydrates, proteins, fats and oils [34–40].

The total alkaloids of the plant extracts were determined by Wagner's reagent. The total carbohydrate in the plant extracts was assessed by Molish Reagent (3.75 g of α-naphthol dissolved in 25 ml of Ethanol 99%). The total reducing sugars were determined by Fehling's solution. Monosaccharides were determined by Barfoed's reagent (copper acetate in distilled water and glacial acetic acid). The amino acids contents of the methanolic extract of the tested plants were determined by Ninhydrin reagent [41] and total protein content was assessed by Folin-Ciocalteau [42]. The terpenoids were assessed by chloroform: sulfuric acid assay, the plant extract was mixed with chloroform, sulfuric acid, and the reddish brown color formation at the interface between the upper chloroform layer and lower sulfuric acid layer confirms the presence of terpenoids. The phytosterols of the plant extract were determined by Liebermann-Burchard's assay. The assay contains methanolic extract of plant (0.5 ml), chloroform (1 ml), acetic acid (1 ml), followed by addition of sulfuric acid. Appearance of pink color which gradually turns into deep green color indicates the presence of sterols.

The flavonoids contents of the plants were detected by ammonia test. The reaction contains plant methanolic extract (0.5 ml) boiled with 10 ml ethylacetate for 3 min, the reaction was filtered, the filtrate was amended with 1 ml of 1% ammonia. Observation of yellowish brown coloration at lower layer of ammonia reveals the flavonoids. The flavonoids were determined by Lead acetate test. The reaction contains plant methanolic extract (1 ml), with drops of lead acetate (10%), the developed yellow precipitate confirmed the flavonoids contents. Also, the flavonoids were detected by Shinoda's test: Magnesium turnings and drops of hydrochloric acid were added to 0.5 ml of the methanolic extract of each plant species. Appearance of crimson red color after 10 min reveals the presence of flavonoids.

The alkaloids were determined by Wagner's test: The reaction contains plant methanolic extract (0.5 ml) warmed with sulfuric acid (2%) for 2 min, followed by addition of Wagner's reagent. The positive result of alkaloid test was confirmed by formation of reddish-brown colored precipitate.

Saponins concentration of the plants were assessed by Foam Test. The reaction contains plant methanolic extract (0.5 ml) combined with distilled water, shaken for 30 min. Formation of a stable honeycomb shaped foam layer persisted for at least 30 min indicates saponins presence.

The tannins titers were quantitatively determined by Braymer's test (Ferric chloride Test). The reaction mixture contains the plant methanolic extract (0.5 ml) with 4 ml of 5% ferric chloride, the development of deep blue color refers to tannins. The phlobatannins concentrations were determined by HCl test. The reaction contains plant methanolic extract (0.5 ml) boiled with 2 ml of conc. HCl for 2 min, and the red precipitate confirms the presence of phlobatannins.

The glycosides concentrations were detected by Keller-Kiliani Test. The methanolic extract of each plant (2 ml) was mixed with glacial acetic acid, followed by addition of 5 ml of 5% ferric chloride, then sulfuric acid (1 ml) was added. Formation of reddish brown ring, with bluish green at the upper surface, indicates the presence of glycosides. The anthraquinone glycosides were determined by hydroxyanthraquinone: Two mls of the methanolic extract of each plant were amended with 5 ml of potassium hydroxide solution (10%), the appeared red color indicates presence of anthraquinone glycosides.

The anthraquinone were determined by Borntrager's test: The assay contains 1 ml of methanolic extract of each plant, 10 ml of diluted ammonia (10%), shaken vigorously for 30 s. The appearance of red solution confirms the presence of anthraquinone. Phenolic compounds were determined by Ferric chloride assay; the methanolic extract of each plant (0.5 ml) was diluted by distilled water, followed by 3 drops of 10% ferric chloride. The appearance of black blue color reveals the presence of phenol compounds. The soluble starch of the tested plants was determined by 5% KOH (1 ml) to the methanolic extract of plant, the reaction was cooled, and then acidified with concentrated sulfuric acid, and the yellow coloration reveals the presence of soluble starch.

The fats and fixed oils were determined by sodium hydroxide test: The extract of each plant (5 drops) was mixed with 2 ml copper sulphate (1%), and sodium hydroxide (10%). Appearance of a clear blue solution indicates the presence of fats and oils. The resins were determined by acetic anhydride test: The methanolic extract for each plant (0.5 ml) was combined with 1 ml of acetic anhydride solution and sulfuric acid, the developed orange to yellow color indicates the resins presence.

The concentrations of coumarins were determined by NaOH test: The plant extract (0.5 ml) was mixed with 1 ml of 10% sodium hydroxide and 1 ml chloroform. Appearance of yellow color confirms the coumarins. Emodins concentration was determined by ammonium hydroxide/benzene test: The plant extract was mixed with 2 ml ammonium hydroxide and 3 ml benzene, and the appearance of a red color confirms the presence of emodins.

The anthocyanin concentration was determined by HCl test: The methanolic extract (0.5 ml) of the plant was mixed with 2 ml of 2 N HCl, followed by 1 ml of ammonia solution. Development of a Pink-red solution which turns blue-violet after addition of ammonia confirms the presence of anthocyanins. The leuconthocyanins concentration was determined by isoamyl alcohol test. The extract (0.5 ml) of each plant was mixed with 1 ml of isoamyl alcohol, the appearance of red upper layer indicates the presence of leuconthocyanins.

The gums and mucilage were qualitatively assessed by alcohol test: The methanolic extract (25 mg) of each plant was dissolved in distilled water (2.5 ml), followed by 2.5 ml of alcohol. The appearance of cloudy precipitate shows the presence of gums and mucilages. The volatile oils were determined by the Fluorescence test: The methanolic extract (2 ml) of each plant was exposed to UV light, as revealed from the development of Bright pinkish fluorescence.

2.4. Quantitative phytochemical analysis

The methanolic extracts of the leaves of *P. gracilior*, *P. elongatus*, *P. macrophyllus* and *P. neriifolius* were used for the quantitative phytochemical assays of their secondary metabolites; terpenoids, flavonoids, phenolic compounds and total antioxidants.

2.4.1. Total terpenoids content (TTC)

The total terpenoids were assessed by sulfuric acid assay [43]. Dried plant extract (0.5 g) was frozen in liquid nitrogen, homogenized in pre-chilled absolute methanol (3.5 ml) with a homogenizer (JRJ300-SH) at 30 Hz for 5 min. After homogenization, the samples were incubated at 30 °C for 48 h in the dark, centrifugation at 4000*g* for 15 min, 0.2 ml of the supernatant were mixed with 1.5 ml chloroform, vortex and kept for 3 min. Sulfuric acid (0.1 ml) was added, and the tubes were incubated at room temperature for 2 h in dark, except the standard solution that was incubated for 5 min. The reddish brown precipitate was collected, and the precipitate was partially soluble in absolute methanol and measured at λ_{538} nm by UV-VIS Spectrophotometer with methanol as blank. The total terpenoids was calculated from the linalool authentic concentrations (Cat. #L2602).

2.4.2. Total flavonoids content (TFC)

Total flavonoids were assessed by aluminum chloride [44,45], the methanolic extract was mixed well with methanol (1.5 ml), aluminum chloride (0.1 ml), potassium acetate (0.1 ml) and water (2 ml), incubated at 30 °C for 30 min. Sample and blank of all extracts were prepared and their absorbance was measured at λ_{415} nm. The flavonoids content was determined regarding to the authentic concentrations of quercetin (Cat. #Q3001).

2.4.3. Total phenolic content (TPC)

The contents of phenolic compounds were assessed by Folin-Ciocalteu assay [46–48], with some modification. Methanolic plant extract was mixed well with 1 ml of Folin-Ciocalteau reagent. After 5 min, 10 ml of sodium carbonate solution (7.5%) was added, mixed, the solution mixture was diluted with water (25 ml) thoroughly, incubation for 90 min at ambient temperature. After incubation, the absorbance of sample was measured at λ_{750} nm. The total phenolic content of the plant samples was measured by the authentic gallic acid (Sigma-Aldrich, Cat. #G7384).

2.4.4. Antioxidant activities

The total antioxidant contents was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [49–51]. The reagent was prepared by dispensing 24 mg of DPPH in absolute methanol (100 ml). The reaction mixture contains 100 μl of the plant methanolic extract to DPPH solution (3 ml). The sample absorbance and the reference compound (ascorbic acid) were measured at λ_{517} nm. The ratio of free radical scavenging activity (RSA) was determined by the formula:

$$\text{RSA}\ (\%) = \frac{\textbf{Absorbance of control} - \textbf{Absorbance of sample}}{\textbf{Absorbance of control}} \times \mathbf{100} \quad (2)$$

The inhibitory concentration (IC_{50}) was expressed by the sample concentration inhibiting 50% DPPH radical scavenging according to the graphic plot of the dose response curve.

2.5. Antimicrobial activity of the plant extract

The biological activity of the methanolic extract of the experimented plants was evaluated against various antibiotic resistant bacteria such as *E. coli*, *P. aeruginosa*, *S. pyogenes* and *S. aureus* [52,53]. The bacterial isolates were identified regarding to their biochemical properties by Bergey's manual [54]. In addition, the antifungal activity of the different plant extracts was assessed against various pathogenic fungi; *Aspergillus flavus, A. fumigatus, A. niger and C. albicans*. The antimicrobial activity of the different extracts was assessed by disc diffusion method [52–55]. The bacterial suspension of 24 h old culture was seeded to the nutrient agar medium, and poured to the sterilized plates. The inoculated plates were incubated for 4 h, then different concentrations of the plant methanolic extracts (15, 30, 45 and 60 μl) were loaded to the filter paper discs to the plate surface. The bacterial cultures were incubated for 37 °C for 24 h, and the fungal cultures was incubated for 5 days at 30 °C. Negative control of methanol was used by the same volumes. Amoxicillin and fluconazole were used as positive controls for bacteria and fungi. The diameters of the inhibition zones were measured in millimeters, and calculated using Image J software portal [52].

2.6. Metabolic profiling of the plant extracts

2.6.1. GC/MS analysis

The metabolic identity of the tested plants methanolic extracts was assessed by GC/MS interfaced with mass selective-detector with polar Agilent HP-5 ms (5%-Phenyl methyl polysiloxane) column (30 m × 0.25 mm × 0.25 μm thickness) and carrier gas "helium", at linear velocity rate 1 ml/min, injector temperature 200 °C and detector temperature 250 °C. The sample (100 μl) was injected, ionization potential 70 eV, interface 250 °C, and acquisition mass range 50–800 *m/z*. The chemical identity of the phytoconstituents was identified according to their spectral fragmentation pattern, and retention times by NIST mass spectral library.

2.6.2. Taxol extraction

Taxol was extracted from the leaves and twigs of *Podocarpus* spp [15]. Parts of the four *Podocarpus* spp (leaves and twigs) were washed and dried at 30 °C, grinded in liquid nitrogen to fine powder. Taxol was extracted according to Whiterup et al. [56], with slight modification. Fifty grams (50 g) of the dried powdered plant tissues was amended with 400 ml n-Hexane for 48 h, centrifuged for 10 min at 5000 rpm to remove the undesirable non-polar compounds. The hexane supernatant solution was carefully discarded, the resulting defatted pellets were frozen at −20 °C overnight, and the pellets were soaked in 200 ml methanol: methylene chloride (1:1) with shaking at room temperature for 24 h, filtered by sterile cheesecloth, and centrifuged at 5000 rpm. The pellets were dried in oven at 60 °C till constant weight, and the combined supernatant extracts from the consecutive extractions were evaporated using rotary vacuum evaporator to separate the solvent from the extract, then kept at −20 °C overnight. To extract Taxol, the dried crude extracts were dissolved in 50 ml methylene chloride and partitioned with 50 ml of distilled water, the two fractions were separated using a separation funnel, the lower layer of methylene chloride fractions (d: 1.3 g/mL) were collected, evaporated till dryness, followed by re-dissolving the dried residues in 5 ml absolute methanol.

2.6.3. Taxol quantification, HPLC and LC/MS analyses

The extracted Taxol was quantified by UV-absorption at wavelength λ_{227} nm [17] using UV-VIS Spectrophotometer compared to authentic one (Cat. #T7402). Methanol was used as negative control for zeroing the spectrophotometer. Standard curve of different authentic Taxol was plotted at λ_{227} nm. The extracted Taxol from each individual *Podocarpus* species was checked by TLC [17],[57]. Ten μl of authentic Taxol and 10 μl of each Taxol extract was spotted on Merck 1 mm, pre-coated silica gel plates with Chloroform: Methanol: Dimethyl formamide (90:9:1 v/v/v) as mobile phase, and the spots of Taxol was detected by illumination at λ_{254} nm.

The proposed spots of Taxol containing silica were scraped-off and suspended in methanol, for Taxol extraction [17]. The amount of Taxol was assessed by HPLC (YOUNG In.) of RP-C18 column (Cat.#959963-902) with methanol: acetonitrile: water (25:35:40, v/v/v) as an isocratic mobile phase at flow rate 1.0 ml/min for 20 min [17]. The identity and concentration of extracted Taxol was confirmed from the retention time and absorption peak at 227 nm [17],[56]. The chemical structure of Taxol was resolved from the LC/MS analysis coupled with a UV and quadruple detector. The mobile phase was water: acetonitrile (10/90) with flow rate of 0.2 ml/min [58,59].

2.6.4. Cytotoxicity of the purified Podocarpus Taxol

The anticancer activity of the putative Taxol sample was assessed towards the liver carcinoma (HepG-2), breast carcinoma (MCF-7) and colon carcinoma (HCT-116), compared to the normal healthy VERO cells, by the MTT assay [60]. The 96-wells plate was seeded with 10^3 cells/well, incubated at 12 h at 37 °C in CO_2 incubator, then adding of different Taxol concentrations, and the plates were incubated for 24 h. The MTT reagent was added, and the intracellular formazan complex was dissolved in DMSO, and the resulted complex was measured at λ_{570} nm. The cell viability was expressed by the sample absorbance per control x100. The IC_{50} value was expressed by Taxol concentration reducing 50% of the initial number of tumor cells compared to saline as baseline.

2.7. Molecular identification of the experimented plants

2.7.1. DNA extraction

The plant genomic DNA was isolated from the dried healthy leaves of *P. gracilior*, *P. elongatus*, *P. macrophyllus* and *P. neriifolius* by Cetyltrimethylammonium bromide (CTAB) [61–63], with minor modification. The dried healthy leaves (0.25 g) were minced to fine powder in liquid nitrogen, transferred to tubes of 500 μl freshly prepared CTAB-β-mercaptoethanol lysis buffer (2x CTAB isolation buffer: 2% CTAB, Tris HCl (100 mM, pH 8.0), EDTA (20 mM), NaCl (1.4 M), PVP40 (2%) and β-mercaptoethanol (0.2%)). The tubes were vortexed, incubated in water bath at 65 °C for 60 min, then centrifuged at 10000 rpm for 10 min at 4 °C, supernatant was mixed with chloroform: isoamylalcohol (24:1), vortex, and centrifuged. The upper aqueous phase was carefully withdrawn and mixed with two volumes of chilled absolute isopropanol and inverted gently several times, then kept at −20 °C for 1 h for DNA precipitation. After sample centrifugation, the DNA pellets were washed by 70% ethanol, the supernatant was discarded, and dried at 30 °C, and 50 μl of TAE buffer was added, and pellets of DNA was kept at −20 °C till further use. The extracted genomic DNA from the *Podocarpus* spp was checked by 1.0% agarose gel (1% agarose) in 1 x TAE (Cat. # AM9864), normalized to 100 bp DNA ladder (Cat. #. DM003-R500 Gene Direx, Inc.). The DNA was visualized by gel documentation system.

2.7.2. PCR amplification and sequencing of the ITS region

The internal transcribed spacer (ITS) region has been used as informative region for phylogenetic comparisons of related species [64,65]. The PCR reaction was performed using the 2 × PCR master mixture (Cat. # 25027) according to the manufacturer's instructions. The amplicons were checked by the 2.0% agarose gel and sequenced by Applied Biosystem Sequencer [18]. The recovered sequence was non-redundantly BLAST searched on NCBI database by ClustalW muscle [66], the phylogenetic relationships were conducted by MEGA version X [67].

2.8. Statistical analysis

The experiments were conducted in biological triplicates, and the results were expressed by the mean ± SD. The significance and F-test were calculated using one-way ANOVA with Fisher's Least Significant Difference of post hoc test.

3. Results and discussion

3.1. Extraction and qualitative phytochemical properties of the different species of Podocarpus

The phytochemical properties of the experimented *Podocarpus* species namely; *P. gracillior*, *P. elongates*, *P. macrophyllus* and *P. neriifolius* were determined. The air-dried plant leaves were pulverized, and the methanolic extracts were prepared. The total weight of the crude extract, dried powder extract and total methanolic extract was shown in Fig. S1. The morphological appearance of the experimented plants were varied from yellow-green (*P. gracillior*), olive green (*P. elongates*) and dark green (*P. macrophyllus* and *P. neriifolius*). The yield of the methanolic extract of *P. gracillior*, *P. elongates*, *P. macrophyllus* and *P. neriifolius* were 41.5% (4.15 g), 11% (1.10 g), 18.7% (1.87 g) and 26.9% (2.69 g), respectively (Fig. S1). The bioactive secondary phytoconstituents were analyzed in the methanolic extracts of the experimented *Podocarpus* species. The total terpenoids, phytosterols, flavonoids, alkaloids, saponins, tannins, phlobatannins, glycosides, anthraquinone glycosides, anthraquinone, carbohydrates, proteins, amino acids, fats and oils were qualitatively estimated. The qualitative detection of the phytochemicals was conducted based on the visual coloration of the reaction. The appearance and intensities of the evolved colored solutions and precipitates, reveals the chemical constituents of leaf methanolic extracts of the investigated plants, as shown on Table 1, and Fig. S2. The methanolic extracts of the plant leaves displayed a fluctuated coloration, revealing the different intrinsic concentrations of the terpenoids, steroids, flavonoids, alkaloids, saponins, tannins, phlobatannins, glycosides, phenolic compounds, monosaccharides, free and combined reducing sugars, fats, oils, coumarins, quinones and volatile oils. But anthraquinine, anthraquinone glycosides, soluble starch, proteins, amino acids, resins, emodins, anthocyanins, leuconthocyanins, gums and mucilages are absent. From the qualitative analysis of bioactive metabolites, *P. neriifolius* had the highest constituents of terpenoids, phytosterols, flavonoids, saponins, tennins, phlobatannins, phenolic compounds, carbohydrates, fats and fixed oils, coumarins, quinones, and volatile oils, followed by *P. macrophyllus* and *P. elongatus*. However, *P. gracilior* exhibited a lower frequency of the tested phytochemical constituents, comparing to the other three experimented plants.

3.2. Quantitative analyses of the phytochemical contents of the tested Podocarpus species

The total bioactive terpenoids, flavonoids, and phenolic compounds were estimated on the experimented *Podocarpus* species. From the profile of phytochemical analysis (Table 1, Fig. 1A,B,C), the highest terpenoids contents were measured for *P. neriifolius* (4.53 mg/g), followed by *P. elongatus* (4.19 mg/g), *P. macrophyllus* (3.98 mg/g), and *P. gracilior* (3.13 mg/g). *P. neriifolius* had the highest concentrations of total flavonoids (23.5 mg/g) and total phenolic compounds (274.3 mg/g), followed by *P. elongatus, P. macrophyllus* and *P. gracilior*. The highest contents of total antioxidants activity was reported for *P. gracilior* (20.7 μg/ml), followed by *P. macrophyllus* (12.7 μg/ml), *P. neriifolius* (10.2 μg/ml) and *P. elongatus* (7.9 μg/ml). From the profile of the phytochemical constituents, *P. neriifolius* had the highest total terpenoids, flavonoids, phenolic compounds and antioxidants activity, followed by *P. elongatus* and *P. macrophyllus*. The fluctuation of the phytochemical constituents of the experimented plants, might be correlated to the environmental and geographical circumstances regulating the molecular expression of the bioactive encoding-gene cluster.

The total antioxidant activities including proteineous and non-proteineous compounds were determined on the methanolic extracts of the tested plants by DPPH. From the profile of antioxidant activity (Fig. 1D and E), the concentration of the antioxidants was increased gradually with the increasing of the plant concentration on a concentration-dependent pattern, with the highest antioxidant activity for *P. gracilior* (20.7 μg/ml), followed by *P. macrophyllus* (12.7 μg/ml), *P. neriifolius* (10.2 μg/ml) and *P. elongatus* (7.9 μg/ml). The higher frequency of the antioxidant activity of *P. gracilior* comparing to other experimented species of *Podocarpus*, authenticate the molecular expression of the antioxidant compounds on the environmental conditions.

3.3. GC/MS metabolic profiling

The metabolic profiling of the experimental plants; *P. gracilior*, *P. macrophyllus*, *P. neriifolius,* and *P. elongatus* were assessed by the GC/MS. Recently, the GC/MS was used as an authenticating technological tool for metabolic outlining, phytochemical constituents and taxonomical purposes [68]. The methanolic extracts of the experimented *Podocarpus* were investigated for their bioactive volatile phyto-constituents by GC-MS. The overall ion current chromatograms of GC–MS results was shown in Figs. 2 and 3, suggesting the presence of various compounds of corresponding peaks at different retention times (Table 2). From the incidence of secondary metabolites as revealed from the metabolic profiling of the experimented plants, *P. gracilior* have the highest frequency of diverse bioactive metabolites (99%), followed by *P. elongatus* (75%), *P. macrophyllus* (60%) and *P. neriifolius* (50%). Total fifty eight

Table 1
Comparative quantification of total phytochemicals of methanol extract of the four *Podocarpus* spp. The methanolic extracts of the tested plants were prepared and their total terpenoids, flavonoids, phenolic compounds and antioxidant activity were determined.

Phytoconstituents	Selected Plant species			
	P. gracilior	P. elongatus	P. macrophyllus	P. neriifolius
Total Terpenoids (mg/g)	3.13 ± 0.79	4.19 ± 0.95	3.98 ± 0.64	4.53 ± 0.71
Total Flavonoids (mg/g)	15.36 ± 0.62	22.67 ± 1.45	15.31 ± 0.95	23.54 ± 1.42
Total Phenolic compounds (mg/g)	140.92 ± 3.64	243.71 ± 5.93	250.56 ± 6.72	274.38 ± 8.19
Total antioxidant activity ($IC_{50,}$ μg/ml)	20.75 ± 3.94	7.90 ± 1.65	12.73 ± 2.19	10.20 ± 1.98

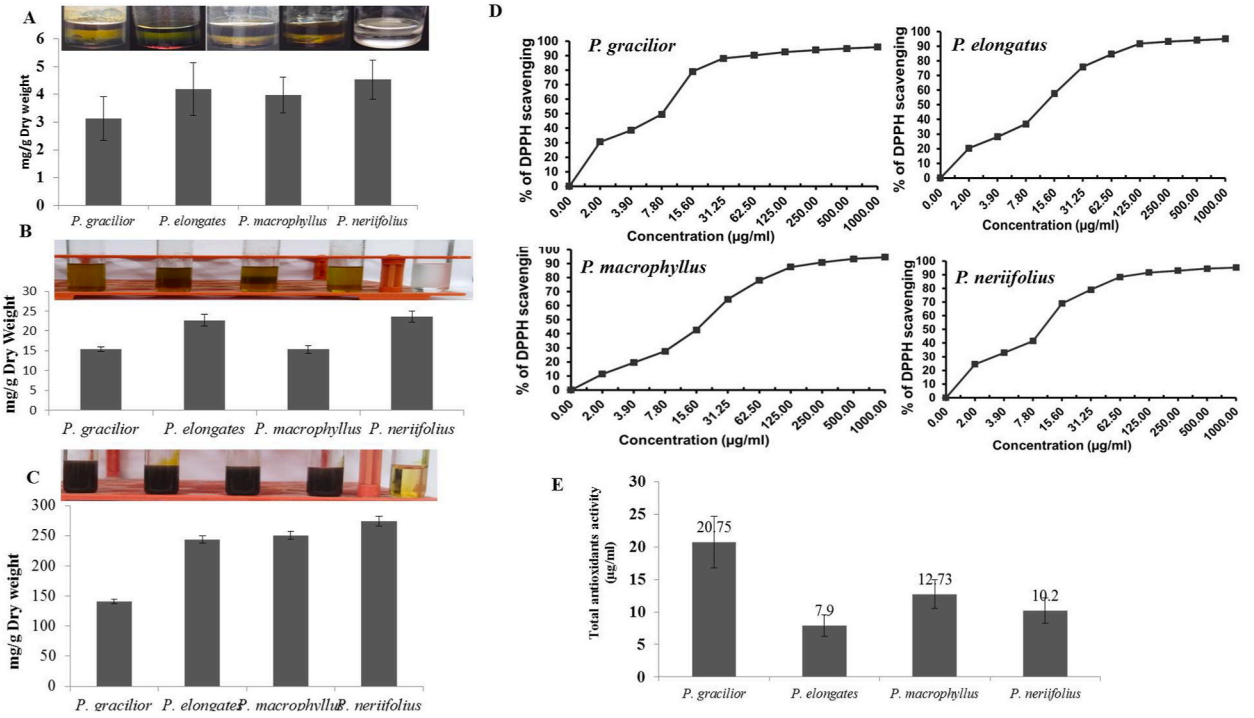

Fig. 1. Quantitative assay of Terpenoids (A), flavonoids (B) and total phenolic compound (C) and total antioxidants (D,E) of *P. gracilior*, *P. elongatus*, *P. macrophyllus* and *P. neriifolius*. The data were represented by means ± STDEV.

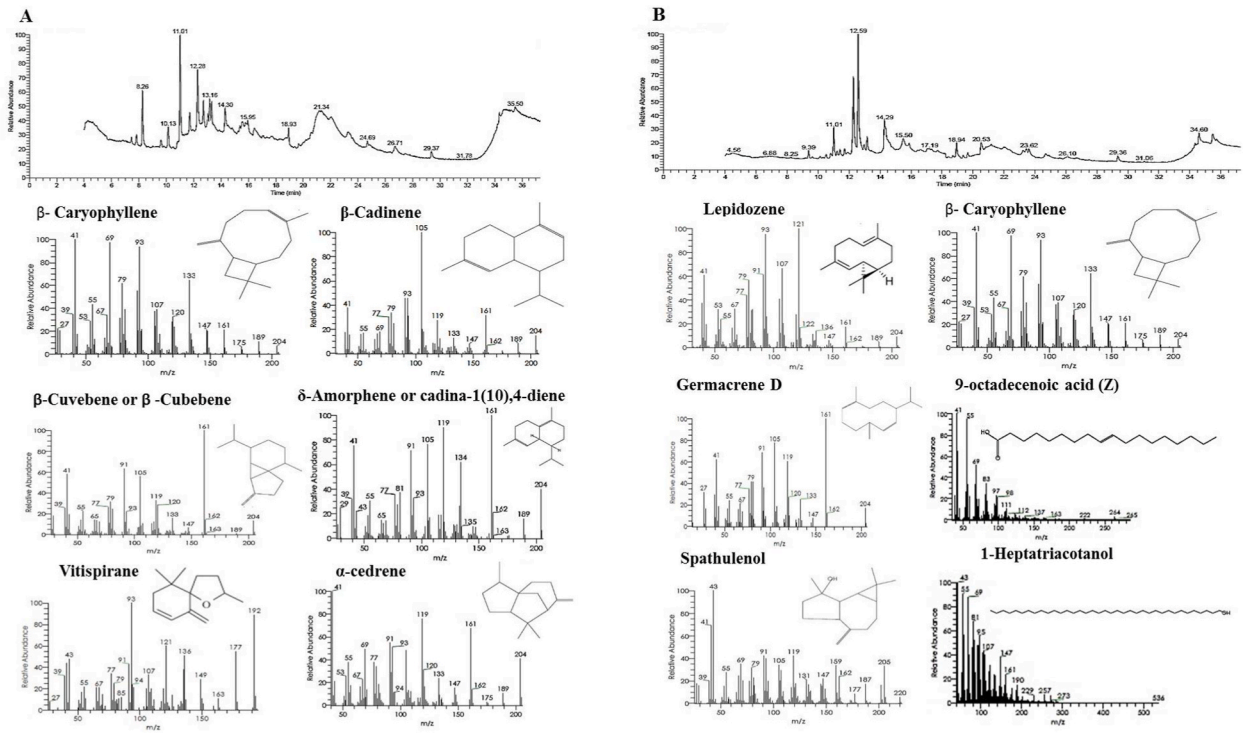

Fig. 2. GC-MS metabolic profiling of the methanolic extracts of *P. gracilor* and *P. elongatus.* The total ion chromatogram (TIC) of the methanolic extract of *P. gracilor* (A) and *P. elongatus* (B). The mass spectra of the most common six metabolites were illustrated under the TIC chromatogram of each plant.

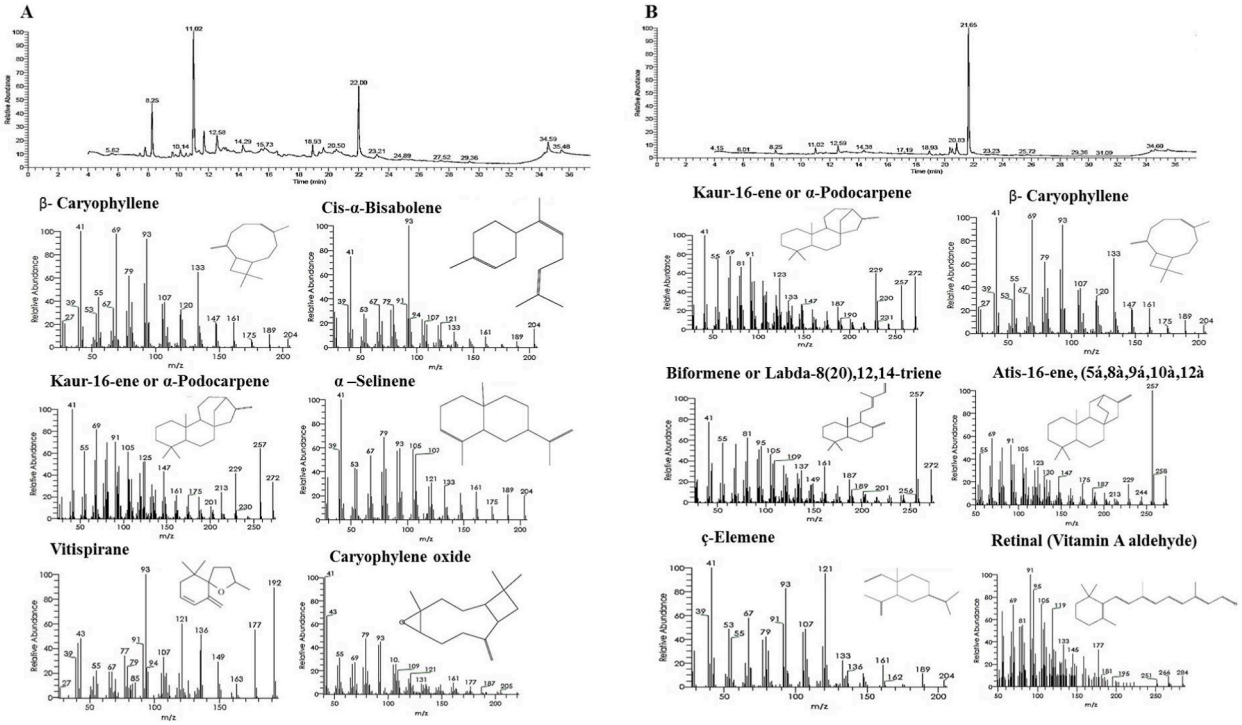

Fig. 3. GC-MS metabolic profiling of the methanolic extracts of *P. macrophyllus* and *P. neriifolius.* The total ion chromatogram (TIC) of the methanolic extract of *P. macrophyllus* (A) and *P. elongatus* (B). The mass spectra of the most common six metabolites were illustrated under the TIC chromatogram of each plant.

compounds were detected in the methanolic extract of the *Podocarpus* species, as revealed the GC/MS profile. From the GC-MS profiling (Table 2, Fig. 2A), the most dominant compound of *P. gracilior* were caryophyllene (17.10%), β-cadinene (4.22%), β-cuvebene (9.16%), vitispirane (8.25%), δ-amorphene or cadina-1(10),4-diene (3.93%) and α-cedrene (3.81%). The most frequent metabolites of *P. elongatus* was lepidozene (26.52%), germacrene (18.27%), spathulenol (10.29%), caryophyllene (7.26%), 9-octadecenoic acid (3.94%) and 1-heptatria-cotanol (3.71%) (Fig. 2B). The most dominant secondary metabolites by *P. macrophyllus* was caryophyllene (36.41%), Kaur-16-ene (18.85%), vitispirane (14.63%), *cis*-α-bisabolenen (6.45%), selinene (5.19%), caryophylene oxide (2.86%). As revealed from the GC-MS results (Fig. 3A), the most incident metabolites of *P. neriifolius,* was Kaur-16-ene (70.95%), labda-8(20),12,14-triene (7.00%), elemene (3.88%), β-caryophyllene (3.66%), atis-16-ene (3.51%), and retinal (1.26%). From the GC/MS profiling, β-caryophyllene was the only compound shared in all of the experimented plants *P. gracilior, P. elongatus, P. macrophyllus* and *P. neriifolius* (Fig. 3B). Practically, among the recovered terpenoids, cubebol, β-cadinene, β-cubebene and δ-amorphene were the most dominant sesquiterpens of *P. gracilior,* unlike to the absence of these compounds on the other *Podocarpus* spp. The unique presence of these metabolites on *P. gracilior,* revealing the molecular manipulation and expression of the sesquiterpens bioactive encoding genes cluster that might be related to the environmental conditions. Similar results for active compounds, antioxidant, podocarpic acid, and other flavonoids, and diterpenoids were extracted from leaves of Podocarpaceae [1]. Many of these active compounds displayed beneficial biological activities, cytotoxic properties, antimicrobial activities [14]. *Podocarpus macrophyllus* bark has been used for treatment of blood disorders, tonic for heart, lungs, kidneys and stomach disorders [12]. The metabolic profiling was used extensively for various taxonomic purposes of the family Taxaceae and Podocarpaceae. The recovered compounds were designated to numerous chemical classes; esters, fatty acids, fatty acid esters, phenolic compounds, alcohols and vitamin E. So, from this study, the phytochemical ingredients of leaves of the experimented species of *Podocarpus* collected from different localities in Egypt were explored. The compound 1,2-benzenedicarboxylicacid, bis(2-ethylhexyl) ester isolated from twigs of *Thevetia peruviana* had a strong anticancer activity on PC3, and MCF-7 [69].

3.4. Antimicrobial activity of the methanolic extracts of the Podocarpus species

The efficacy of the different methanolic extracts of *P. gracilior, P. elongatus, P. macrophyllus* and *P. neriifolius* was assessed by disc diffusion assay, against the multidrug resistant bacteria; *E. coli*, *P. aeruginosa, S. pyogenes* and *S. aureus,* as well as, against fungal isolates *Aspergillus fumigatus*, *A. niger* and *C. albicans.* From the antibacterial profile (Fig. 4A), the methanolic extracts of the plants displayed an obvious activity towards the tested bacterial isolates in a concentration-dependent manner, comparing to methanol as negative control. The inhibition zone diameter of *P. gracilior* methanolic extract (60 μl) for *E. coli*, *P. aeruginosa*, *S. pyogenes* and *S. aureus* were ranged between 20 mm and 25 mm. As well as, the antibacterial activity of *P. neriifolius* was maximally reported towards

Table 2
Comparative GC-MS metabolic profiling of the methanolic extracts of the experimented *Podocarpus* species, showing the predicted compound name, molecular formula and their identities.

	RT (min)	Compound	Nature of Compound	Molecular formula	Selected Plant species (Peak area %)			
					P. gracilior	P. elongatus	P. macrophyllus	P. neriifolius
1	4.09	Linoleic Acid Chloride	Omega-6 fatty acid	$C_{18}H_{31}ClO$	0.96	–	–	–
2	4.16	2-(7-Heptadecynyloxy) tetrahydro-2H-pyran	Flavonoid	$C_{22}H_{40}O_2$	0.87	–	–	–
3	4.26	Oleic acid	Omega-9 fatty acid	$C_{18}H_{34}O_2$	1.22	–	–	–
4	7.45	Oxamyl	Carbamate ester	$C_7H_{13}N_3O_3S$	1.18	–	–	–
5	7.82	2,5,5,8A-tetramethyl −3,5,8,8 A- tetrahydro-2H-chromene	C13-apocarotenoids or Norisoprenoids	$C_{13}H_{20}O$	1.94	–	–	–
6	8.26	Vitispirane	C13-apocarotenoids or Norisoprenoids	$C_{13}H_{20}O$	8.25	–	14.63	1.99
7	9.39	ç-Elemene	Sesquiterpenoid	$C_{15}H_{24}$	–	1.98	5.19	3.88
8	9.60	Farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol)	Acyclic sesquiterpene alcohol	$C_{15}H_{26}O$	1.46	–	–	–
9	9.61	α-copaene	Sesquiterpenoid	$C_{15}H_{24}$	–	–	1.91	–
10	10.13	α-cedrene	Sesquiterpenoid	$C_{15}H_{24}$	3.81			
11	10.80	2-Methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butenal	Aldehyde	$C_{14}H_{22}O$	–	1.14	–	–
12	10.81	2,3-Dehydro-4-oxo-α-ionol	Sesquiterpenoid	$C_{13}H_{18}O_2$	0.65	–	–	–
13	11.01	β- Caryophyllene	Bicyclic sesquiterpenoid	$C_{15}H_{24}$	17.10	7.26	36.41	3.66
14	11.71	*Cis*-α-Bisabolene	Sesquiterpenoid	$C_{15}H_{24}$	3.21	–	6.45	–
15	12.28	β-Cuvebene or β –Cubebene	Sesquiterpenoid	$C_{15}H_{24}$	9.16	1.12	–	–
16	12.58	α –Selinene	Sesquiterpenoid	$C_{15}H_{24}$	–	–	5.19	3.88
17	12.59	Lepidozene	bicyclogermacrene sesquiterpenoid	$C_{15}H_{24}$	–	26.52	–	3.88
18	12.70	β-Cadinene	Sesquiterpenoid	$C_{15}H_{24}$	4.22	–	–	–
19	13.03	α-ylangene	Sesquiterpenoid	$C_{15}H_{24}$	1.62	1.51	2.12	–
20	13.16	δ-Amorphene or cadina-1(10),4-diene	Sesquiterpenoid	$C_{15}H_{24}$	3.93	3.64	–	–
21	13.31	Cubebol	Sesquiterpenoid	$C_{15}H_{26}O$	3.49	–	–	–
22	14.28	Spathulenol	Sesquiterpenoid	$C_{15}H_{24}O$	–	10.29	–	–
23	14.29	Caryophylene oxide	sesquiterpenoid alcohol	$C_{15}H_{24}O$	0.75	–	2.86	–
24	15.37	α-acorenol	Sesquiterpenoid	$C_{15}H_{26}O$	0.63	1.79	–	–
25	15.49	Cedrane-8,13-diol	Sesquiterpenoid	$C_{15}H_{26}O_2$		3.53	–	–
26	15.54	Longifolene or Junipene	Tricyclic sesquiterpenoid	$C_{15}H_{24}$	1.52	–	–	–
27	15.78	2,5-Octadecadiynoic acid, methyl ester	Fatty acid	$C_{19}H_{30}O_2$	0.74	–	2.94	–
28	15.85	2-[4-Methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	Aldehyde	$C_{23}H_{32}O$	0.75	–	–	–
29	15.89	10,12-Pentacosadiynoic acid methyl ester	Fatty acid	$C_{26}H_{44}O_2$	–	1.33	–	–
30	15.95	Methyl 4,7,10,13-hexadecatetraenoate	Fatty Acid	$C_{17}H_{26}O_2$	1.66	–	–	–
31	16.43	Retinal (Vitamin A aldehyde)	Vitamin	$C_{20}H_{28}O$	3.18	–	–	1.26
32	18.92	9-octadecenoic acid (Z)	Fatty Acid	$C_{18}H_{34}O_2$	–	3.94	3.67	2.49
33	18.94	12-Methyl-E,E−2,13-octadecadien-1-ol	Fatty alcohol	$C_{19}H_{36}O$	2.05	–	–	–
34	19.65	Vincadifformine	Alkaloid	$C_{21}H_{26}N_2O_2$	–	–	1.80	–
35	19.66	1-Heptatriacotanol	Fatty alcohol	$C_{37}H_{76}O$	–	1.33	–	–
36	20.36	Atis-16-ene, (5á,8à,9á,10à,12à	Diterpenoid	$C_{20}H_{32}$	–	–	–	3.51
37	20.52	Palmitic acid, methyl ester	Fatty acid	$C_{17}H_{34}O_2$	–	3.50		
38	20.54	Tetraacetyl-*d*-xylonic nitrile	Sugar acid	$C_{14}H_{17}NO_9$	1.11	–	–	–
39	20.83	Biformene or Labda-8(20),12,14-triene	Diterpenoid	$C_{20}H_{32}$	–	–	–	7.00
40	20.99	Desulphosinigrin	Carbohydrate	$C_{10}H_{17}NO_6S$	2.31	–	–	–
41	21.06	Melezitose	Carbohydrate	$C_{18}H_{32}O_{16}$	2.01	–	–	–
42	21.15	3-*O*-Methylhexose (3-*O*-Methyl-*d*-glucose)	Carbohydrate	$C_7H_{14}O_6$	4.31	–	–	–
43	21.99	Kaur-16-ene or α-Podocarpene	Diterpenoid	$C_{20}H_{32}$	–	–	18.85	70.95
44	22.09	2,3-Dihydroxypropyl palmitate	Fatty acid ester	$C_{19}H_{38}O_4$	1.48	–	–	–
45	22.18	Dodecanoic acid, 2,3-bis(acetyloxy) propyl ester	Fatty acid ester	$C_{19}H_{34}O_6$	1.03	–	–	–
46	23.26	Pentadecanoic acid	Fatty acid	$C_{15}H_{30}O_2$	0.61	–	–	–
47	23.30	cis-Oxiraneundecanoic acid	Fatty acid	$C_{19}H_{36}O_3$	1.01	–	–	–
48	23.42	3-Oxo-20-methyl-11-à-hydroxyconanine-1,4-diene	Steroid alkaloid	$C_{22}H_{31}NO_2$	0.80	–	–	–

(*continued on next page*)

Table 2 (*continued*)

	RT (min)	Compound	Nature of Compound	Molecular formula	Selected Plant species (Peak area %)			
					P. gracilior	P. elongatus	P. macrophyllus	P. neriifolius
49	23.61	Methyl stearate	Fatty acid	$C_{19}H_{38}O$	–	1.70	–	–
50	24.68	Methyl-9,9,10,10-D4-octadecanoate	Fatty acid	$C_{19}H_{34}D_4O_2$	0.99	–	–	–
51	26.73	9,10 dideutero octadecanoic acid	Fatty acid	$C_{18}H_{34}D_2O_2$	1.45	–	–	–
52	29.36	10-Methoxy-N(*b*)-à –methylcorynantheol	Alkaloid	$C_{21}H_{29}N_2O_2$	1.46	1.72	–	–
53	34.19	2-Monolinolenin 2TMS derivative	Fatty acid	$C_{27}H_{52}O_4Si_2$	0.35	–	–	–
54	34.34	Calcitriol or 1,25-Dihydroxyvitamin D3, TMS derivative	Vitamin	$C_{30}H_{52}O_3Si$	1.29	1.02	–	0.83
55	34.58	Vitamin E, alpha-tochopherol	Vitamin	$C_{29}H_{50}O_2$	–	–	3.18	–
56	34.66	Quercetin 7,3′,4′-trimethyl ether	Flavenoid	$C_{18}H_{16}O_7$	0.76	3.03		1.58
57	35.48	1-Heptatriacotanol	Fatty alcohol	$C_{37}H_{76}O$	–	3.71	–	–
58	35.50	Ergosta-5,22-dien-3-ol, acetate, (3á,22E)-	Steroid	$C_{30}H_{48}O_2$	1.05	–	–	–

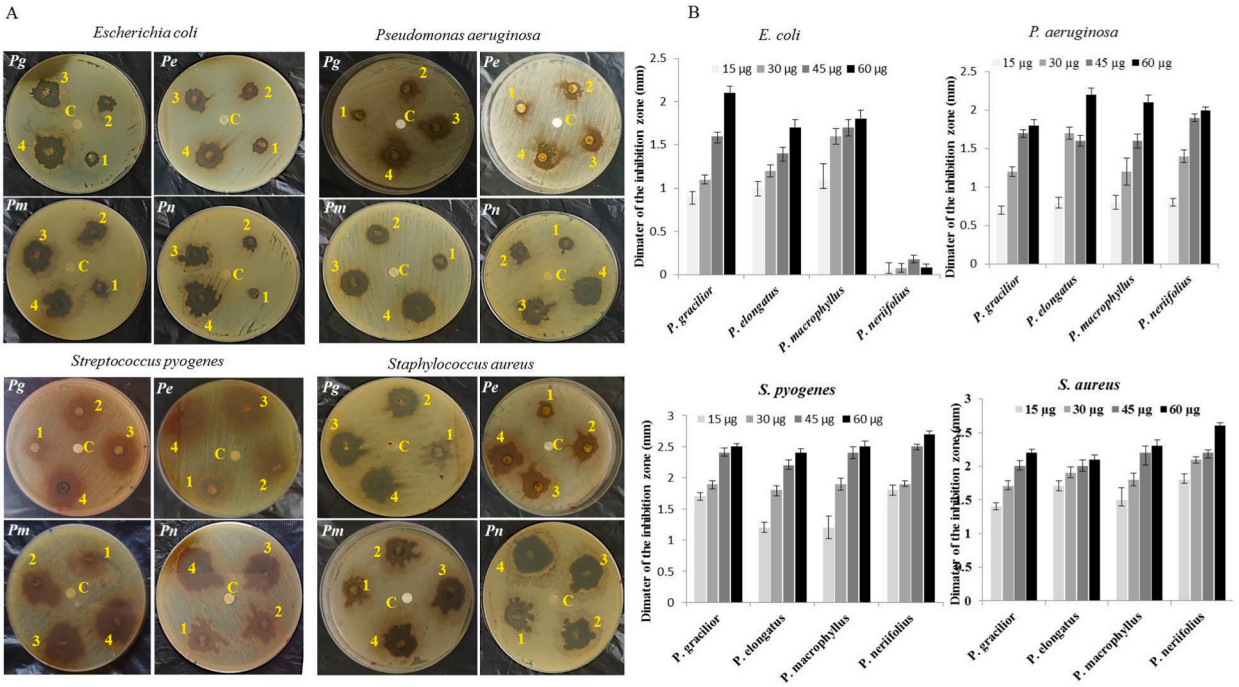

Fig. 4. Antibacterial activity of the methanolic leaf extracts of *Podocarpus* spp towards the multiple drug resistant bacteria; *E. coli*, *Pseudomonas aeruginosa, Streptococcus pyogenes* and *Staphylococcus aureus* by disc diffusion assay. *P. gracillior* (Pg); *P. elongatus* (Pe); *P. macrophyllus* (*Pm*) and *P. neriifolius* (Pn). Different concentrations of the plant methanolic extracts were used (1 (15 µl), 2 (30 µl), 3 (45 µl) and 4 (60 µl)), injected to the Whatman Filter paper disk, uniformly placed on the surface of bacterial plate culture and incubated at 3 days at 37 °C. B, The diameter of the inhibition zones the different bacterial isolates in response to the response to plant extracts. The data were represented by means ± STDE, with *p*-value ≤0.05 (ONE Way ANOVA test).

E. coli, *P. aeruginosa*, *S. pyogenes* and *S. aureus* with inhibition zone ranged between 20 and 28 mm, comparing to methanol as negative control. The antimicrobial efficiency of the tested extracts was assessed from the IC_{50} values towards the different bacterial isolate (Fig. 4B). The antifungal activity of the methanolic extract of the experimented plants was assessed towards *A. fumigatus*, *A. niger* and *C. albicans*. From the antifungal activity profile (Fig. 5A), the extracts of *Podocarpus* species displayed a strong activity towards *A. fumigatus*, *A. flavus* and *A. niger*. The highest antifungal was reported for the extracts of *P. neriifolius* towards *A. fumigatus*, *A. niger* and *C. albicans* with average inhibition zone 20–25 mm, at 60 µl of the plant extract (Fig. 5B). Remarkably, the antimicrobial activity of the plant extract towards the tested fungal isolates was increased in a concentration-dependent manner, revealing the significant impact of the bioactive constituents of the plant extracts.

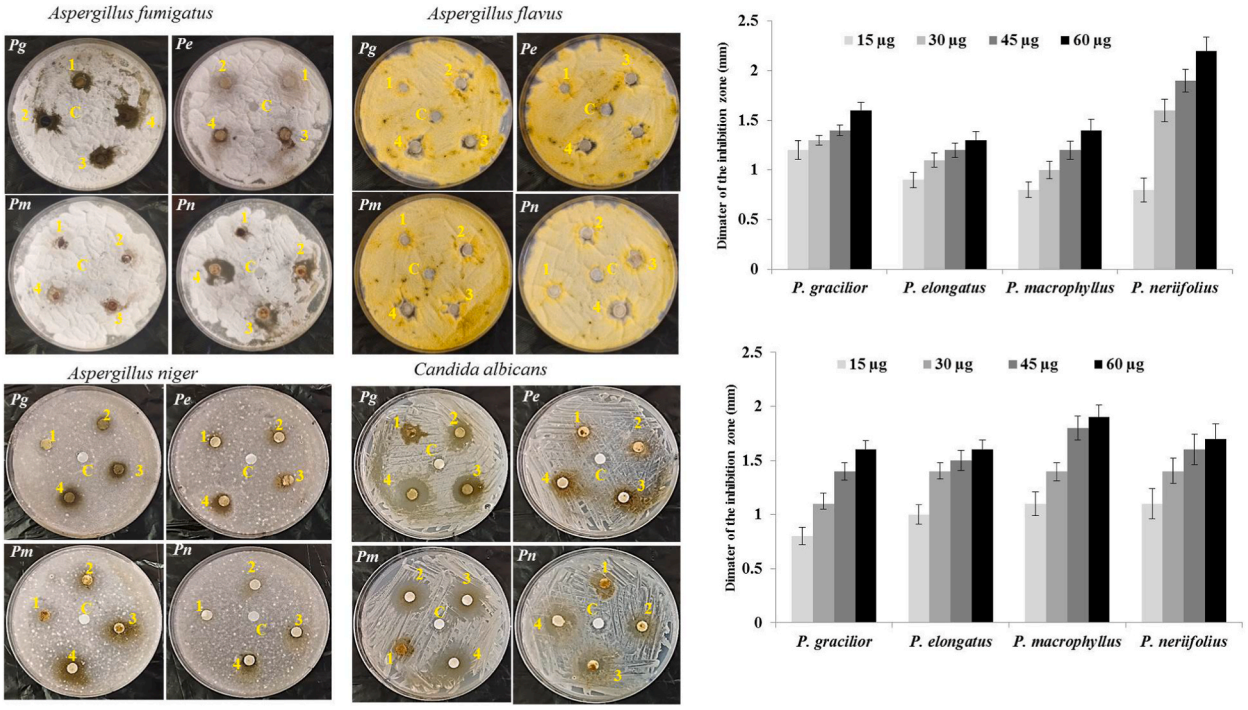

Fig. 5. Antifungal activity of the methanolic leaf extracts of *Podocarpus* spp against the different phytopathogens *Aspergillus fumigatus*, *A. flavus*, *A. niger* and *C. albicans P. gracillior* (*Pg*); *P. elongatus* (*Pe*); *P. macrophyllus* (*Pm*) and *P. neriifolius* (*Pn*). Different concentrations of the plant methanolic extracts were used (1 (15 µl), 2 (30 µl), 3 (45 µl) and 4 (60 µl)), injected to the Whatman Filter paper disk, uniformly placed on the surface of 5 h old fungal plate culture, incubated for 5 days at 30 C. B, The diameter of the inhibition zones the tested fungal isolates in response to the response to plant extracts were measured. The data were represented by means ± STDE, with *p*-value ≤0.05 (ONE Way ANOVA test).

3.5. Taxol extraction, chemical identification and antiproliferative activity from the Podocarpus species

Taxol as powerful bioactive compound was extracted and determined from the experimented *Podocarpus* species. The plant samples were grinded in liquid nitrogen, amended with n-hexane, dissolved in methylene chloride, and Taxol was extracted by the solvent systems (Fig. 6A). From the TLC profile, a noticeable fluctuation on the yield of Taxol was reported from the different *Podocarpus* species (Fig. 6B). The yield of Taxol was chemically verified from the HPLC chromatogram (Fig. 6C, Fig. S3), regarding to the concentration and retention time of authentic Taxol at 27–2.9 min. The maximum Taxol yield was detected on the extracts of *P. elongatus* (16.4 µg/g dry weight), *P. macrophyllus* (11.2 µg/g dry weight), and *P. neriifolius* (8.6 µg/g dry weight). Based on the productivity, the Taxol sample extracted from *P. elongatus* has been used for further chemical verification analyses. The crude Taxol extracts from *P. elongatus* has been further purified by the preparative-TLC. The structural identity of the extracted Taxol from *P. elongatus* was confirmed by LC/MS analysis. Taxol of *P. elongatus* had the same molecular mass to charge ratio (854.6 *m/z*), and the same molecular fragmentation pattern of the authentic one, as revealed from the LC-MS/MS analysis (Fig. 7A). Similarly, LC/MS has been used for verification of the chemical structure of the small bioactive metabolites of microbial and plant origins [41]. Consistently, Taxol has been structurally authenticated from *Ozonium* sp [70] by the LC/MS approach. Similar results validate the chemical identity of Taxol from plant and fungal sources implementing the identical approaches of chromatography and spectroscopy [17,18].

3.6. The antiproliferative activity of extracted Taxol from the selected Podocarpus species

The anticancer activity of the purified Taxol from *P. elongatus* was verified towards various cell lines namely liver carcinoma (HepG-2), breast carcinoma (MCF-7), and colon carcinoma HCT-116 by MTT assay (Fig. 7B). The antitumor activity of the Taxol was evaluated based on viability of tumor cells, as well as, the IC_{50} values. From the anticancer activity, the extracted Taxol from *P. elongatus* had the highest activity towards the HCT-116 (IC_{50} value 30.2 µg/ml), followed by HepG-2 (53.7 µg/ml) and MCF-7 (71.8 µg/ml). The antitumor efficiency of the purified Taxol from *P. elongatus* was very consistent to Taxol from *Taxus brevifolia* [70], and from *Aspergillus terreus* [18], and *A. flavipes* [52]. As well as, the antitumor efficiency of the purified Taxol from *P. elongatus* being consistent with Taxol from *N. sylviforme* and *C. oxysporum* extracted Taxol [71]. The cytotoxic activity of *A. terreus* Taxol against HEPG2 and MCF7 tumor cells was higher than Taxol from *T. brevifolia* (590–762 nM).

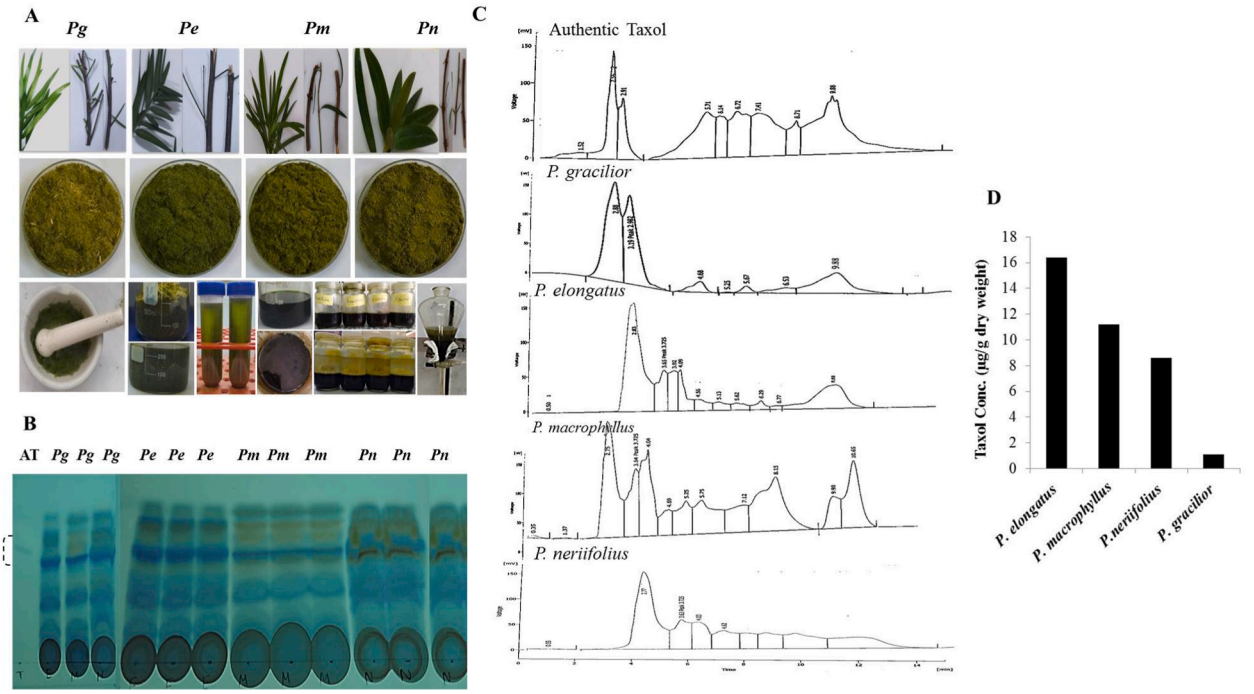

Fig. 6. Taxol extraction and quantification from the tested *Podocarpus* species. A, Overall view of plant maceration and Taxol extraction from the tested plants. B, TLC profile of Taxol from the experimented plants. C, HPLC chromatogram of Taxol extracted from the different plants and the authentic one. D, Taxol concentration based on the HPLC chromatogram compared to the authentic concentration of Taxol. The data were represented by means ± STDE, with *p*-value ≤0.05 (ONE Way ANOVA test).

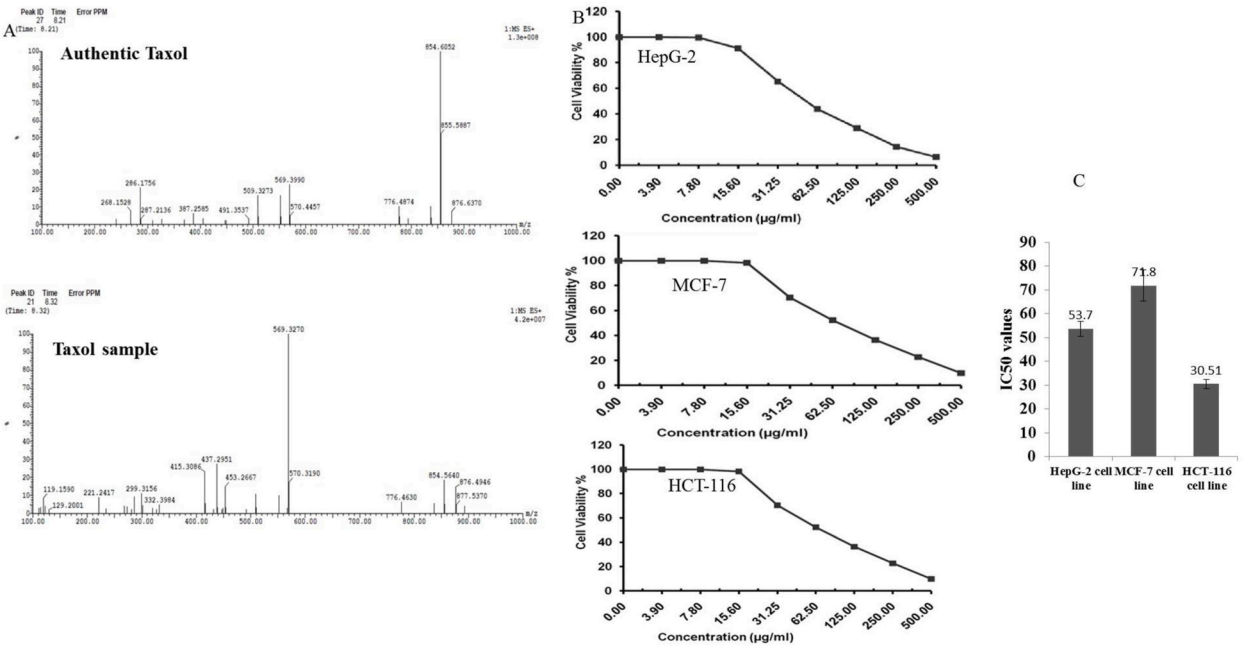

Fig. 7. LC/MS chromatogram and antiproliferative activity of the extracted Taxol from *P. elongatus.* A, LC/MS chromatogram of authentic Taxol and putative sample. B, antiproliferative activity of extracted Taxol from *P. elongatus* towards different tumor cell lines HepG-2, MCF-7 and HCT-116. C, IC_{50} values of Taxol for the tested cell lines.

3.7. Molecular identification of the potent Taxol producing Podocarpus species

The ITS sequence region has been used as a molecular marker for identification of the plant species [68]. Using the genomic DNA as PCR template for amplification of the ITS regions, the size of PCR product was about 650 bp (Fig. 8A and B). The PCR products were sequenced and non-redundantly BLAST searched on NCBI database. From the multiple alignment analysis, the ITS sequence of *P. elongatus* EFBL-NZM was deposited on Genbank with accession # ON540734.1, that is the first record for *Podocarpus* species on Genbank. The phylogenetic analysis of the ITS sequence of *P. elongatus* has been constructed by MEGA-X software package with the Maximum Likelihood method. From the phylogenetic analysis of ITS region of *P. elongatus*, two clades were evolved; Clade I "*Bauhinia* group" and Clade II "*Taxus* group" (Fig. 8C). The ITS sequence of *P. elongatus* displayed 99.5% similarity with the ITS regions of *Bauh*inia blakeana AF3879701, B. variegate AY258378.1, B. purpurea KX057836.1, B. acuminata JX856404.1, B. monandra KX057835.1, B. faberi AF390195.1, B. brachycarpa FJ432276.1, B. ungulata FJ009818.1, B. cheilantha DQ787410.1, B. jennigsii AY258411.1, B. macranthera JN942381.1, B. rufescens KX057837.1, and C. chingii JQ425130 with zero E-value and 100% query coverage. While, the sequence of the ITS region of *P. elongatus* displayed a 60% similarity with the ITS sequence of different *Taxus* species; *Taxus walichiana* MH711827.1, *T. cuspidata* MK123471.1, *T. brevifolia* MK1234701.1, *T. canadensis* MK211148.1, *T. x media* MK123472.1, *T. canadensis* MK272738.1, *T. cuspidata* MK168616.1 and MK123473.1.

4. Conclusion

Although the myriad pharmaceutical applications of the bioactive metabolites of the different species of Podocarpaceae, a few studies reporting the phytochemical constituents, and metabolic profiling of the *Podocarpus* species inhabiting Egypt. Thus, different species of *Podocarpus* "*P. gracilior, P. elongatus, P. macrophyllus* and *P. neriifolius"* were collected from the different geographical locations in Egypt, and their metabolic profiling and phytochemical constitutes were analyzed comparatively. *Podocarpus gracilior* being the reservoir of the bioactive metabolites especially terpenoids, followed by *P. elongatus*, *P. neriifolius* and *P. macrophyllus.* The methanolic extracts of *P. elongatus* gave the highest activity against *E. coli*, *P. aeruginosa*, *S. pyogenes* and *S. aureus,* as well as the *Aspergillus fumigatus*, *A. niger* and *C. albicans.* The bioactive diterpenoids "Taxol" has been extracted and quantified from the experimented *Podocarpus* species, *P. elongatus* have the highest concentration of Taxol, followed by *P. macrophyllus, P. neriifolius* and *P. gracilior.* The anticancer activity of Taxol derived from *P. elongatus* was authenticated on different tumor cell lines (HepG-2, MCF-7 and HCT-116), that being similar to commercial Taxol from *T. brevifolia.* The highest Taxol encompassing *P. elongatus* has been molecularly identified as the first record and deposited on Genbank with accession number ON540734.1. However, the main limitation of this study was the tiny yield of Taxol, as well as the availability of these plants. Thus, further experimental trials on using the tissue culture approaches of *P. elongatus* of Taxol production are ongoing by our research group.

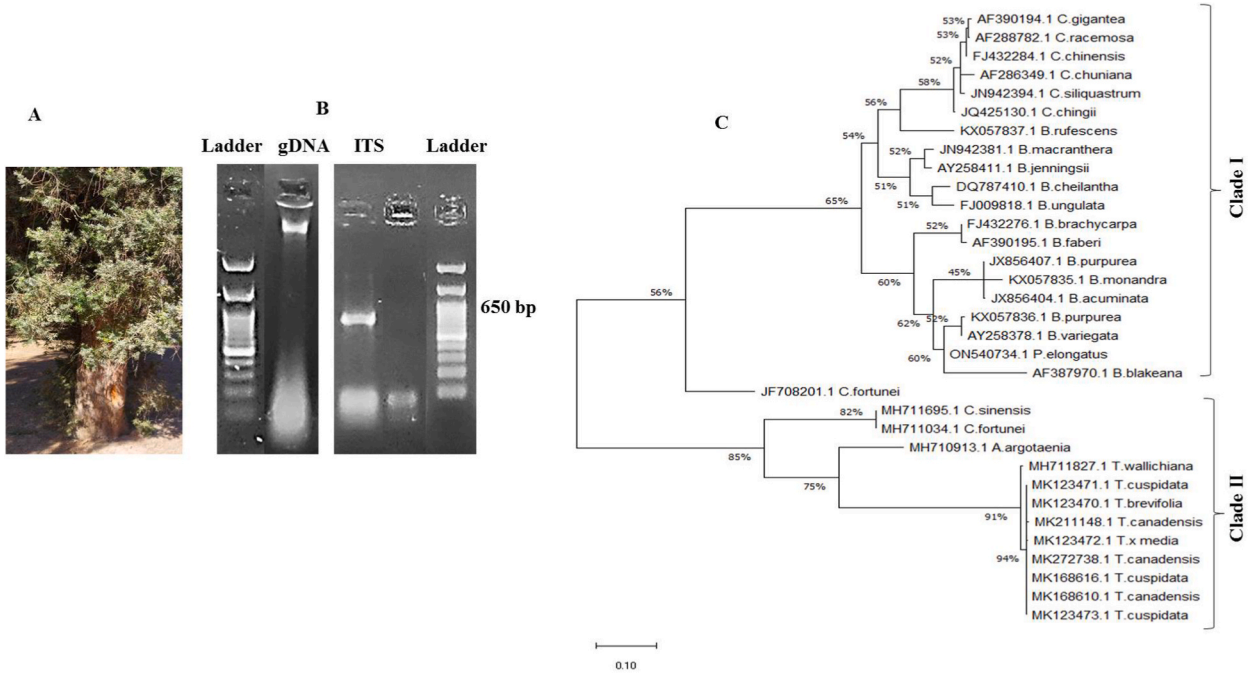

Fig. 8. Morphological view and molecular identification of the potent Taxol producer plant "*Podocarpus elongatus"*. A, Morphological view of *Podocarpus elongatus*, showing the entire tree, and leaves. The plant was molecularly identified based on the sequence of the ITS region of the rDNA. The genomic DNA was extracted and used as PCR template. B, Plant genomic DNA and PCR amplicons of the ITS region with molecular size about 650 bp. C, The phylogenetic tree of the ITS region of *P. elongatus* using the MEGAX software package with Maximum likelihood.

5. Author contributions statements

1 - Conceived and designed the experiments;
2 - Performed the experiments;
3 - Analyzed and interpreted the data;
4 - Contributed reagents, materials, analysis tools or data;
5 - Wrote the paper.

Ethical standards

This article does not contain any studies with human participants or animals.

Availability of data and materials

All the data are available in the manuscript.

Declaration of competing interest

The authors declare that they have no competing of interests.

Acknowledgement

The authors are also very much thankful and grateful to Trease Labib, a plant taxonomist at Orman Garden, Giza, Egypt, who shared her knowledge on identification of the plant materials, and confirmation of the *Podocarpus* species.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20034.

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