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Original Article

Bioactive Natural Products from *Polyalthia Longifolia* and its Fungal Endophyte *Alternaria sp.*

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ABSTRACT

Medicinal plants and fungi are rich sources of natural products. The plant *Polyalthia longifolia* was investigated for its phytochemical components and its own endophytes. Phytochemical investigation of the aerial parts of *P. longifolia* has led to the isolation and identification of *β*-sitosterol-3-*O*-*β*-D-glucoside (**1**), 14,15-bisnor-3,11-*E*-kolavadien-13-one (**2**), 3-*β*,5-*β*,16-*α*-trihydroxyhalima-13(14)-en-15,16-olide (**3**), isoquercetin (**4**), rutin (**5**), and *trans*-cinnamic acid (**6**). Furthermore, the endophyte *Alternaria sp.* (MT995125) was isolated from the leaves and the compounds pyrenolide-D (**7**) and modiolide-A (**8**) were identified in its solid culture. The isolated compounds were tested against human epithelioid cervical carcinoma (HeLa), human colorectal carcinoma (HCT-116) and human amnion (WISH) normal cell line. The compounds showed moderate to strong cytotoxic activity with minimal toxicity towards normal cell line WISH compared to the positive control doxorubicin. The significant cytotoxic activities of some compounds were augmented by *in silico* strong inhibitory activity to carbonic anhydrase (IX isoform) (CA IX) through performing docking experiment.

***Keywords*:** *Polyalthia longifolia;* Fungal endophyte; Clerodane diterpenes; *Alternaria*; Cytotoxicity; Carbonic anhydrase.

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INTRODUCTION

Polyalthia longifolia, family Annonaceae, is a lofty evergreen tree native to India and Sri Lanka. It is introduced in gardens in many tropical countries around the world, for example its cultivation in Tanzania, Indonesia and the Caribbean islands not only for ornamental purposes but also to combat noise and dust. The plant genus *Polyalthia* is characterized chemically by the production of terpenoids (mainly diterpenes), alkaloids, steroids, polyphenols, acetogenins, and flavonoids [1]. There are over 100 species of *Polyalthia* sp. distributed from Africa to Asia and the Pacific [2]. They have shown marked activity as antimicrobial, anti-inflammatory, cytotoxic, immunosuppressive, antibacterial, antifungal, anti-malarial and other pharmacological properties [3,4].

Endophytic fungi are found virtually in almost every plant and living organism as symbiotic or slightly pathogenic microorganisms [5,6] and they are prolific and sustainable producers of bioactive natural products with interesting structures [7]. Worldwide, colorectal and cervical cancers were the third and fourth most common cancer among women, respectively [8]. Recent epidemiologic studies revealed a high incidence of colon and cervical cancer among young Egyptian population [9]. Carbonic anhydrase IX enzyme plays an important role in the growth and survival of tumor cells under normoxia and hypoxia, making it a potential target for colon and cervical cancer therapy [10].

The purpose of this study was to investigate the chemical constituents of the methanolic extract of the aerial parts of *P. longifolia* and of the ethyl acetate extract of *Alternaria* sp. solid culture (Fungal endophyte isolated and identified for the first time from *P. longifolia* fresh leaf).

On the other hand, we measured the cytotoxic activities of the isolated compounds on two cancer cell lines: human epithelioid cervical carcinoma (HeLa), human colorectal carcinoma (HCT-116). Their cytotoxicity was determined using normal cell line human amnion (WISH). Besides, the binding affinity of the isolated compounds to carbonic anhydrase (IX isoform) (CA IX) active site amino acids residues was evaluated by performing docking experiment for them using acetazolamide as standard reference CA inhibitor.

MATERIAL AND METHODS

General: UV spectra (λ_{max}) was carried out on UV-visible spectrophotometer (Shimadzu 1601 PC, model TCC-240A, Japan) using spectroscopic methanol. IR spectra (cm^{-1}) was carried out on FTIR spectrometer, Thermo Fisher Scientific, Nicolet is 10 (USA) using KBr pellets, all in central lab, Faculty of Pharmacy, Mansoura University. Nuclear Magnetic Resonance spectra (^{1}H-NMR, ^{13}C-NMR, DEPT-Q, APT, HSQC, HMBC, COSY and NOESY) were obtained on a Bruker DRX 600 NMR spectrometer (600 and 150 MHz for ^{1}H and ^{13}C-NMR, respectively) in Japan or on a Bruker Avance III 400 spectrometer (400 and 100 MHz for ^{1}H and ^{13}C-NMR, respectively) in NMR unit, Faculty of Pharmacy, Mansoura University. The solvents used are CD_3OD, $CDCl_3$ and DMSO-d_6. Mass spectra were obtained on LC/MS/MS UPLC API 4000 (Sciex, Concord, Ontario, L4K 4V8, Canada) in Faculty of Pharmacy, Ain-Shams University, or on compact mass spectrometer TLC/MS (Advion, USA) in Nawah scientific, Cairo, Egypt. Normal phase chromatography was carried out using silica gel G 60-230 mesh (Merck, Germany) packed by the dry method or the wet method in the specified solvent.

Plant material: The plant material used in this study consists of aerial parts of *P. longifolia* (Annonaceae) was collected from Dar-ElSalam (Tanzania), during January 2017, and identified by Dr. Ibrahim Mashaly, Professor of Ecology, Faculty of Science, Mansoura University, Egypt. A voucher specimen "01-17-PL-Mansoura" was deposited at the Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University.

Endophytic fungal material: Two fresh leaves of *P. longifolia* were cut into very small pieces, washed with sterilized water, and then the surface was thoroughly sterilized with 70% ethanol for 1-2 minutes and air dried under the flow hood to avoid surface contaminating microbes. After this process, leaf fragments were inoculated in Petri dishes containing Malt agar (MA) medium and incubated for five days till the fungal hyphae almost covered the surface of the malt agar plate. The individual strains were isolated by transferring hyphae tips growing out with a sterile loop onto a fresh malt agar dish. For purification of the fungal strains, this step was repeated several times until the colony was uniform. The isolated strain was identified according to a molecular biological protocol by DNA amplification and sequencing of the ITS region as described by Kjer *et al.* [11]. The GenBank accession number of the fungus is (GenBank: MT995125). The name of the strain is *Alternaria sp.* PLTW.

Extraction and isolation procedures:

A- For the plant: The fresh plant material (2 kg) was chopped into small pieces, dried at room temperature and then soaked in methanol for 3 days. The methanolic extract was evaporated under

reduced pressure giving a viscous residue (500 g). This was dissolved in methanol-water (1:1) then successively extracted in separating funnel till exhaustion by petroleum ether (5 X 2 L), methylene chloride (5 X 2 L) and ethyl acetate (5 X 2 L). Each extract was then concentrated under reduced pressure to yield petroleum ether fraction (100 g), methylene chloride fraction (95 g) and ethyl acetate fraction (25 g).

A-1. Isolation of compounds: Methylene chloride extract (90 g) was applied onto the top of a glass column (120 x 5 cm) packed with two layers of fine silica and silica gel (250 g each). The column was then gradiently eluted with petroleum ether-methylene chloride (until 100% methylene chloride) then with methylene chloride-methanol (100:0 to 0:100). Collected fractions were subjected to purification through chromatography and repeated crystallization to give compounds **1** (3 mg) and **2** (3 mg) in pure form.

Ethyl acetate extract (25 g) was applied onto the top of a silica gel column gradiently eluted with petroleum ether-ethyl acetate (100:0 to 0:100) then with increased proportions of methanol in ethyl acetate till 100% methanol. Collected fractions were subjected to purification through chromatography and repeated crystallization to afford compounds **3** (20 mg), **4** (30 mg), **5** (30 mg) and **6** (4 mg) in pure form.

A-2. Identification of the isolated compounds: Six compounds were isolated from *Polyalthia longifolia* methanolic extract. Compounds **1-6** (Figure 1) were identified by comparing their data with chemical, physical and spectroscopic published data in literature [12-16]. These compounds are *β*-sitosterol-3-*O*-*β*-D-glucoside **1**, 14,15-bisnor-3,11-*E*-kolavadien-13-one **2**, 3-*β*,5-*β*,16-*α*-trihydroxyhalima-13(14)-en-15,16-olide **3**, isoquercetin **4**, rutin **5**, and *trans*-cinnamic acid **6**.

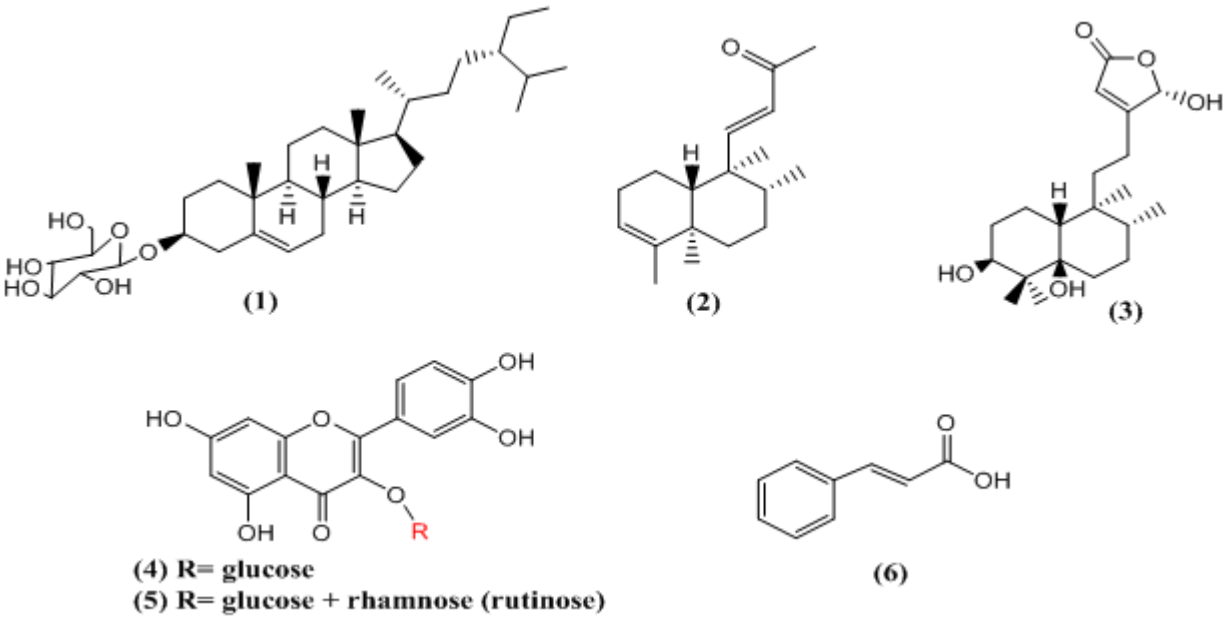

Figure (1) Structures of natural compounds 1-6 isolated from *Polyalthia longifolia*

B- For the endophyte: A solid medium was prepared by autoclaving fifteen 1L Erlenmeyer flasks, with 100 g white rice and 120 ml distilled water in each flask. Fungal cultures were transferred from petri-dishes to flasks (1 petri-dish for each flask) under sterile conditions and closed with sterile cotton plug. Fermentation was allowed for 4 weeks at room temperature away from light [17].

Fungal metabolites in each flask were extracted thrice with ethyl acetate. The combined ethyl acetate extract was washed with little distilled water, filtered and then evaporated under reduced pressure till dryness. The dry residue (50 g) was dissolved in 50% methanol and fractionated using *n*-hexane, methylene chloride and ethyl acetate in a separating funnel to yield 990 mg, 510 mg and 540 mg respectively, while the remaining methanol extract yielded 30 g.

B-1. Isolation of compounds: Ethyl acetate extract (540 mg) was applied onto the top of a silica gel column (10 g, 35 x 1 cm) previously packed in petroleum ether. The extract was then gradiently eluted with methylene chloride-methanol (100:0 to 0:100) then with increased proportions of methanol in ethyl acetate till 100% methanol. Collected fractions were subjected to purification through chromatography and repeated crystallization to

give compounds **7** (15 mg) and **8** (15 mg) in pure form.

B-2. Identification of the isolated compounds: Two compounds were isolated for the first time from the solid culture of *Alternaria sp.* ethyl acetate extract. Compounds **7** and **8** (Figure 2) were identified by comparing their data with chemical, physical and spectroscopic published data in literature [18-20]. These compounds are pyrenolide-D **7** and modiolide-A **8**.

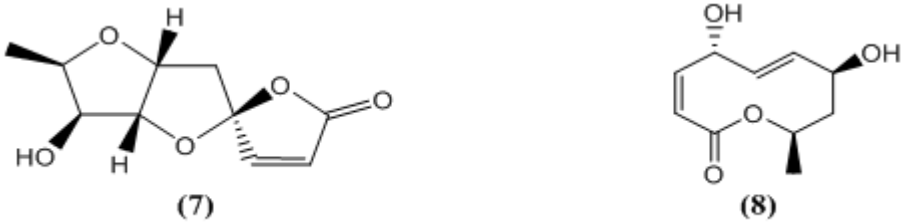

Figure 2: Structures of natural compounds **7** & **8** isolated from Alternaria sp. endophyte of *Polyalthia longifolia*

Cell line and culture conditions: Human colorectal carcinoma (HCT-116) and human epithelioid cervical carcinoma (Hela) cell lines in addition to human amnion (WISH) normal cell line were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt, they were maintained in RPMI-1640 medium containing 10% FBS and 1% pen-strep and incubated at 37°C in 5% CO_2 / 95% humidity.

MTT cytotoxicity assay: This colorimetric assay is based on the reduction of the pale yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a purple formazan derivative by mitochondrial succinate dehydrogenase enzymes in viable cells. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100 µg/ml streptomycin at 37 ^{0}C / 95% humidity in a 5% CO_2 incubator. The cell lines were seeded in a 96-well plate at a density of $1.0x10^4$ cells/well. at 37 ^{0}C for 48 h under 5% CO_2.

After incubation the cells were treated with different concentration of compounds and incubated for 24 h. After 24 h of drug treatment, 20 µl of MTT solution at 5mg/ml was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl was added into each well to dissolve the purple formazan formed.

The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified by colorimetric assay at absorbance of 570 nm using a plate reader (ELISA reader, EXL 800, USA). The relative cell viability in percentage was calculated as (A_{570} of treated samples/A_{570} of untreated sample) X 100.Compounds having cytotoxic activities cause mitochondrial dysfunction consequently a decrease in the color intensity [21,22].

Carbonic anhydrase IX enzyme in silico inhibition: The neutral forms of docked compound structures were built using Molecular Operating Environment (MOE version 2009.11) builder (Chemical Computing Group Inc. software. https://www.chemcomp.com). The MMFF94 force field program was used for carrying out the conformational analysis with root mean square (RMS) gradient of 0.01 kcal/mol Å. The isolated compounds were docked into the binding site of CA (IX) enzyme (PDB code ID: 3IAI) [23]. Target CA (IX) enzyme preparation for docking process was performed by adding the hydrogen atoms, then refining the target structure by adding the missed bonds and atoms, and finally fixing the enzyme potential. The active site was defined as the region of CA (IX) which comes within 10 Å from the ligand [24]. Visualization of all docking files was performed using MOE software.

RESULTS AND DISCUSSION

MTT cytotoxicity assay: In the present study, the cytotoxic activities of the isolated compounds were shown against the mentioned cancer and normal cell lines through the colorimetric assay (Table 1).

Compound **4** showed the highest cytotoxic activity against HCT-116 & HeLa cells. Compound **5** showed lower cytotoxic activity against both cells, these results were compatible with the reported cytotoxic activities for such compounds [13,14]. Compound **2** showed moderate cytotoxic activity against HCT-116 & HeLa cells. Compound **3** showed stronger cytotoxic activity against both cells, while compound **1** showed weak cytotoxic activity towards HCT-116 & HeLa cells.

Compound **7** showed moderate cytotoxic activity against HCT-116 & HeLa cells, while compound **8** showed weak cytotoxic activity against both cells. Cytotoxicity of compounds **7** and **8** against HCT-116 & HeLa cells is reported for the first time. Compounds **4** and **5** were relatively safer towards

normal cell line WISH than the positive control doxorubicin suggesting potential candidates for developing high safety margin cytotoxic agent for treatment of colon and cervical cancer.

Table (1):IC_{50} (µg/ml) values of the cytotoxic activity of different compounds isolated from *P. longifolia* and its endophyte *Alternaria* sp. on HCT-116, HeLa, and WISH cell lines.

Comp.	In vitro Cytotoxicity IC_{50} (µg/ml)•		
	Normal cell line	Cancer cell lines	
	WISH	HCT-116	HeLa
•• DOX	3.18±0.2	5.23±0.3	5.57±0.4
1	80.10±4.7	62.53±3.9	59.23±3.8
2	70.15±4.3	55.07±3.6	47.73±3.4
3	25.00±2.0	37.71±2.8	40.25±2.9
4	33.79±2.6	10.88±0.9	9.63±0.7
5	43.03±3.1	22.62±1.8	15.08±1.3
6	N.t.*	N.t.*	N.t.*
7	45.43±3.2	28.08±2.3	31.86±2.5
8	66.49±4.1	77.22±4.5	51.73±3.5

• IC_{50} (µg/ml): 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak) and above 100 (non-cytotoxic); •• DOX: Doxorubicin; * N.t.: Not tested

Carbonic anhydrase IX enzyme in silico inhibition: The conformational analysis and the binding interaction of the isolated compounds with CA (IX) enzyme pocket were performed in comparison to acetazolamide [25] as standard reference CA inhibitor using MOE program. The docking results and bonding types are shown in Table 2. The interaction of acetazolamide (Fig. 3a) to CA (IX) is shown as a complex binding. Regarding the strongest cytotoxic isolated compounds **4, 5 & 7**, they shared acetazolamide in strong binding interactions with various amino acid residues. These compounds had a better docking score than the reference drug, while other less active compounds have comparable docking score to the reference drug (Fig. 4).

Table (2): The average cytotoxic activity (IC_{50}µg/ml), docking scores[a] and type of binding interactions of the isolated compounds and the reference compound (acetazolamide).

Comp-ounds	Cytotoxicity (IC_{50} µg/ml) **	Binding energy (Kcal/mol) (docking score)	Type of binding interactions
1	67.29	-6.60	Strong hydrophobic interaction with Thr200, Gln67, Pro72 & Leu91
2	57.65	-6.96	Strong hydrophobic interaction with Thr200, Leu198, His94 & His64
3	34.32	-6.99	Metal chelation with His119 using Zn^{2+} metal; Strong hydrophobic interaction with Thr200, His94, His64 &Gln67
4	18.1	-13.55	Two H-bonds with Trp5 & His64; Two Metal chelation bonds with His119 using Zn^{2+} metal; Strong hydrophobic interaction with Thr200, Leu198, His94 & Gln92
5	26.91	-10.44	Three H-bonds with Arg58, Gln67 & Gln92; Metal chelation with His119 using Zn^{2+} metal; Arene-arene interaction with His94; Strong hydrophobic interaction with Thr200, Leu198, His64 & Val131
6	N.t.***	N.t.***	N.t.***
7	35.12	-8.43	Two H-bonds with His64 & Thr200; Metal chelation with His119 using Zn^{2+} metal; Strong hydrophobic interaction with His94 & Gln67
8	65.15	-6.43	Strong hydrophobic interaction with Thr200, Leu198 & His94
Aceta-zolam-ide*	-	-6.75	Two H-bonds with Thr200 & His94; Arene-arene interaction with His94; Metal chelation with His119 using Zn^{2+} metal; Strong hydrophobic interaction with Leu198 & Gln67

* Acetazolamide were used as positive controls.

** All data are presented as mean value ± SD for three independent experiments. *** N.t.: Not tested

[a] Docking was performed using MOE 2009.10 towards the active site of CA (IX) (code: 3IAI) (RMS gradient of 0.01 kcal/Å mol).

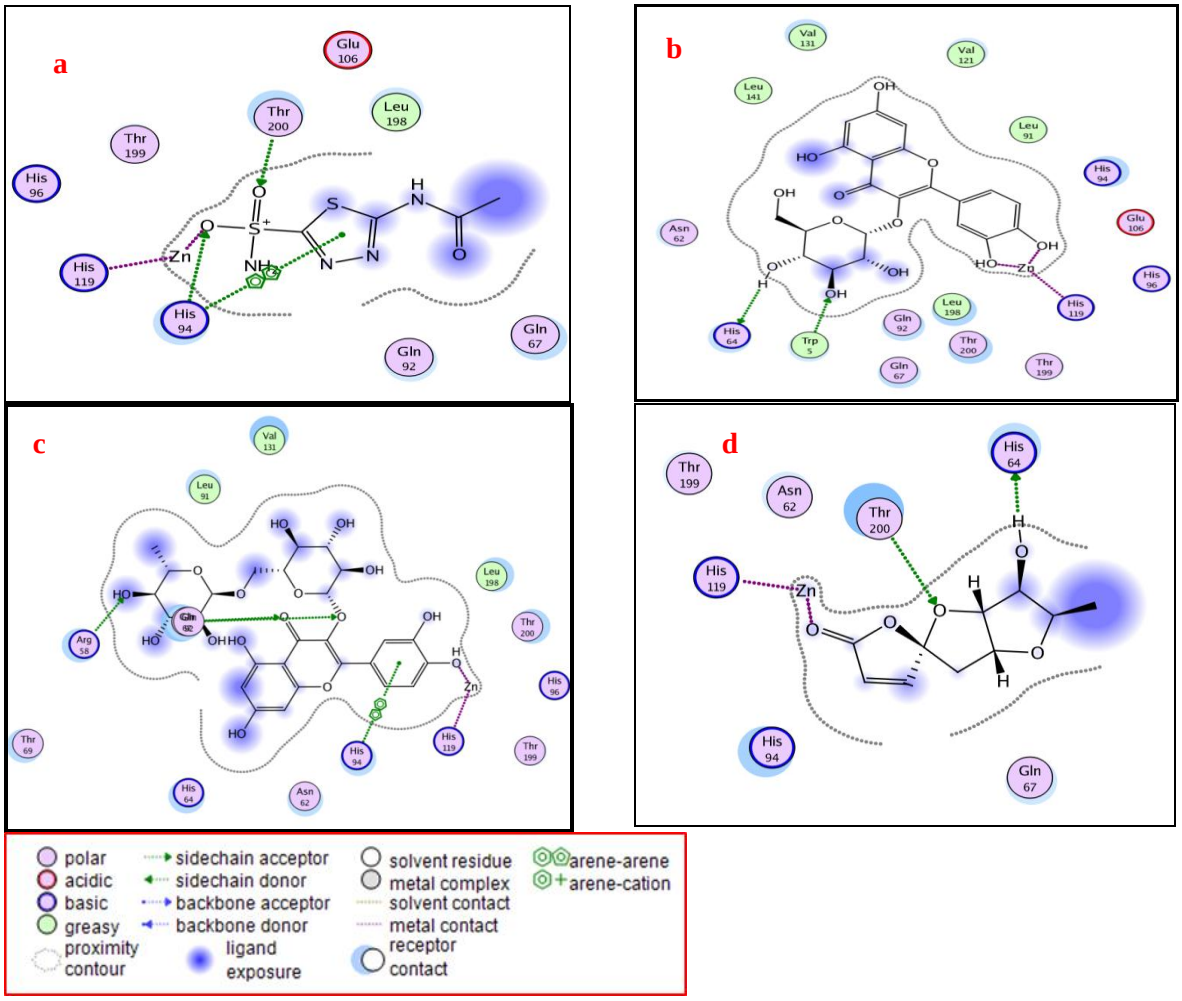

Figure (3) 2D binding mode of acetazolamide (a) and the strongest cytotoxic compounds 4 (b), 5 (C) and 7 (d) docked and minimized in the CA (IX) binding pocket.

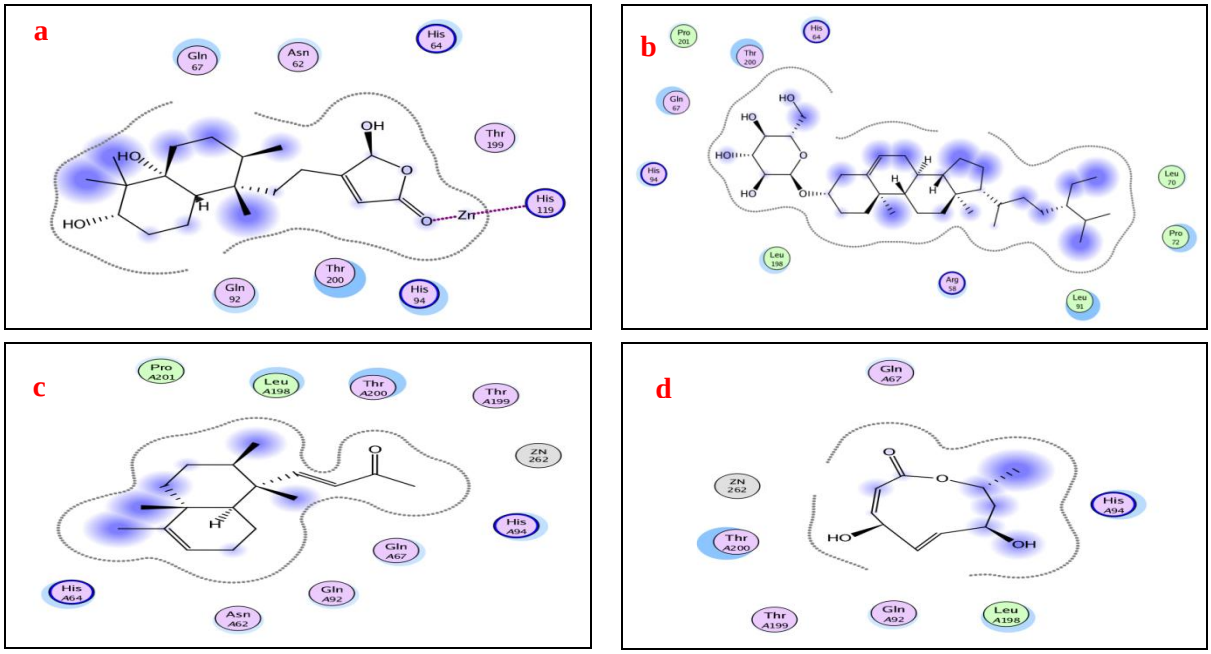

Figure (4) 2D binding mode and residues involved in the recognition of the moderately active cytotoxic compound 3 (a) and the least active cytotoxic compounds 1 (b), 2 (C), and 8 (d) docked and minimized in the CA (IX) binding pocket.

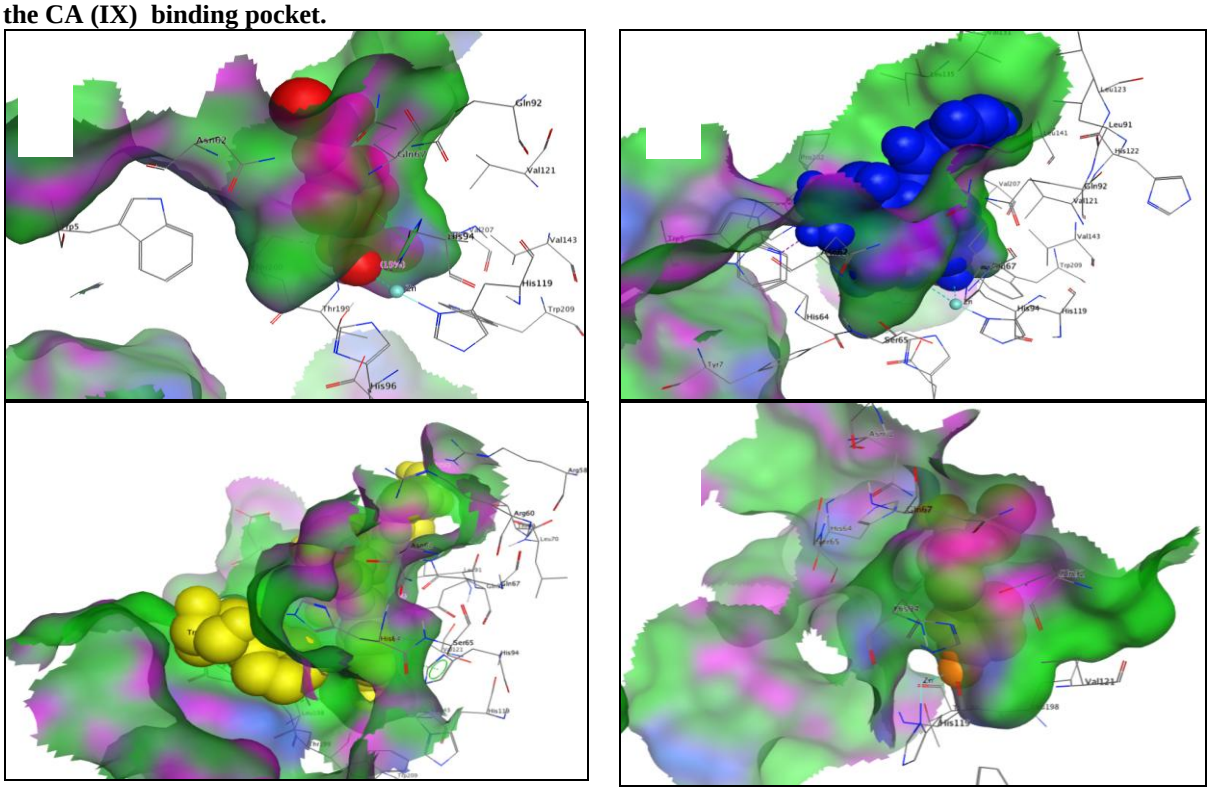

Figure (5): The aligned conformations of (a) acetazolamide (space filled red) and the strongest cytotoxic compounds 4 (b) (space filled blue), 5 (C) (space filled yellow) and 7 (d) (space filled orange) occupying the CA (IX) binding pocket surface map.

The unique binding interaction of compound **4** with CA (IX) is shown in Fig. 3b, while in compound **5** (Same skeleton of **4** with extra rhamnose**)** (Fig. 3c), it exceeds acetazolamide by forming 3 more H-bonds. Three-dimensional docking results visualization was also performed to simulate the ligands binding to CA (IX) active site. The binding pocket surface mapping showed that the strongest cytotoxic isolated compounds **4, 5 & 7** filled and occupied the whole space of the CA (IX) binding pocket in similar manner to that of acetazolamide (Fig. 5)**.**

CONCLUSION

The present study showed that the metabolites from *P. longifolia* and its fungal endophyte *Alternaria* sp. (isolated for the first time from its leaves) could be potential candidates for developing high safety margin cytotoxic agent for treatment of colon and cervical cancer. These activities can be partly attributed to compounds **3**, **4**, **5** in addition to **7** as they shared the highest *in vitro* and *in silico* cytotoxic and CA (IX) inhibitory activities among the isolated compounds.

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