SUPPLEMENTRY MATERIAL

A new firewall in the fight against breast cancer: *In-vitro* and *in-silico* studies correlating chemistry to apoptotic activity of *Otostegia fruticosa*

Somaia. A. Al-Madhagy[a,b], Sameh S. Gad[c], Eman S. Mostafa[d], Simone Angeloni[e,f], Muhammed A Saad[g,h], Omar M. Sabry[a,i]*, Giovanni Caprioli[e], Seham S. El-Hawary[a]

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ABSTRACT

Breast cancer is the most devastating disease for women. There is a great demand for new sources to treat this disease. Medicinal plants are an indispensable source of bioactive compounds with wide range of pharmacological activities. *In-vitro* cytotoxic activity of *Otostegia fruticosa* methanolic extract against human breast cancer was studied using MCF-7 cell line. The extract showed mildly potent activity (IC_{50}= 51 ± 9.836 µg/ml) in comparison to the standard anticancer doxorubicin (IC_{50} = 7.467 ± 1.05 µg/ml). Potential compounds responsible for activity have been identified using Molecular Operating Environment (MOE) module on the major compounds detected by HPLC-MS/MS technique against estrogen alpha receptor (ERα+: PDB ID 2JF9). 3,5-di-*O*-dicaffeoylquinic acid, hyperoside and rutin showed similar binding and antagonistic interaction with the estrogen alpha receptor as tamoxifen in several poses. The retrieved results confirm that we can add this plant to a powerful arsenal that combats this insidious disease.

Keywords:

MCF-7, *Otostegia fruticosa*, HPLC-MS/MS, Phenolics, estrogen alpha receptor

Experimental

Plants materials

As mentioned in our previous investigation to make Parkinson's disease extinct (Al-Madhagy et al. 2022), *Otostegia fruticosa* leaves were collected in July 2020 form Bani Matar village, Sana'a Governate, Yemen. Plant materials were kindly authenticated by Dr. Abdul Wali Ahmed Al-Khulaidi, Associate Professor of flora and vegetation at the Agricultural Research and Extension Authority "Southern Upland Station" Taiz/Ibb, Yemen. The authenticated plant was deposited as a voucher specimen at the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, with the voucher number 13-10-2021I. The collected samples were air dried in the shed before being stored in tightly sealed containers until use.

Extraction

The dried leaves (10 gm) of *Otostegia fruticosa* were subjected to ultrasonic assisted extraction using 90% HPLC grade methanol. The extract was filtered, dried using rotary evaporator at 40ºC, and sent for investigation.

Chemicals and Reagents

Chemicals used in HPLC-MS/MS analysis was mentioned before in our previous work (Al-Madhagy et al. 2022). For cytotoxicity MTT assay, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, RPMI-1640 medium, Dimethyl sulfoxide (DMSO), Phosphate-buffered saline (PBS) and MCF7 Cell Line were purchased from (Sigma-Aldrich, Germany), while MTT solution was purchased from (Bio Basic Inc., Canada) (Lewandowski et al. 2022).

HPLC-ESI-MS/MS Technique

Detailed HPLC-MS/MS methodology was mentioned before in our previous work (Al-Madhagy et al. 2022). Figure **S4** shows the HPLC-MS/MS chromatogram of standard mixture of 30 bioactive compounds plotted as overlapped MRM transition. Table **S2** displays the 14 bioactive components, expressed in µg/g of dried weight extract, monitored in the *Otostegia fruticosa* methanolic extract.

Cytotoxicity activity (MTT assay) (Lewandowski et al. 2022)

Cytotoxic activity of *Otostegia fruticosa* methanolic extract was tested using MTT assay test. Briefly, a 96 well tissue culture plate was inoculated with 1 x10^5 cells/ mL (100 μL /well) and incubated at 37°C for 24 hours to develop a complete monolayer sheet. Growth medium was decanted from the 96 well microtiter plates after confluent sheet of cells were formed and cell monolayer was washed twice with wash media. Six concentrations of *Otostegia fruticosa* methanolic extract (1000, 500, 250, 125, 62.5, 31.25 μg/mL) were prepared using RPMI medium and 2% maintenance medium (serum). 0.1 ml of each dilution was tested in different wells leaving 3 wells

as control (maintenance medium only). Plate was incubated again at 37°C. Cells were examined and checked for any physical signs of toxicity, such as partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. 20μL MTT solution (5mg/ml in PBS) were added to each well and mixed thoroughly using shaking table (150 rpm for 5 minutes). The plates were incubated at 37 ºC and 5% CO_2 for 4 hours to allow the MTT to be metabolized. Media was dumped off and MTT metabolic product (formazan) was resuspended in 200 μL DMSO and shacked on a shaking table (150 rpm for 5 minutes). Optical density was read at 560 nm and background was subtracted at 620 nm. The relative cell-viability percentage was evaluated by using the following relation:

$$\text{Relative cell-viability \%} = \frac{\text{Absorbance of cells treated with the tested sample at 560 nm}}{\text{Absorbance of untreated cells at 560 nm}} \times 100$$

In-silico study (Durcik et al. 2022)

The X-ray generated structure (PDB ID: 2JF9) of estrogen receptor (ER) complexed with the biological metabolic product of tamoxifen, 4-hydroxytamoxefine (4-OHT), was chosen for molecular docking study due to good experimental resolution (2.10), R-value free and R-value work (0.198 and 0.180, respectively). Before running the molecular docking, we validated our docking protocol by retrieving 4-OHT from the crystallographic ER structure and docking it into the binding site again to ensure that the docking program could reproduce the antagonist bioactive conformation of 4-OHT. In this validation step, the co-crystalized ligand had a root mean square deviation (RMSD) of 0.5581 in comparison to the original X-ray derived conformation, the best pose depicted in Figure **S5** shows that 4-OHT formed hydrophobic interactions between its butenyl group and aromatics rings and receptor's aromatic rings, as well as a positive ionizable interaction and hydrogen bonds between its amino group, and the phenoxy and hydroxyl oxygens of the receptor.

Protein ligand docking was accomplished using the induced fit pose generation method. Before docking process, the tested compounds were configured by removing water molecules, adding hydrogen atoms, addition of atomic charges, adjustment of potential energy and adjusting other critical parameters using the MMFF94x force field. The compounds were then prepared for the docking step in MDB format. Protein structure was prepared by removing water molecules and other non-protein-related heteroatoms, adding of hydrogen atoms and selecting of the binding domain of the receptor's surface. The energy-score for each pose was estimated upon 30 trails using London dG scoring function that improved twice by triangle Matcher methods before it was final refined using GBVI/WSA dG score function. The criteria of considering H-bonding to be effective is to mustn’t exceed 3.5 Å.

Statistical Analysis

Results of MTT cytotoxic activity assay were expressed as a mean of three experiments ± standard deviations (S.D.). Inhibitory concentration 50 for the extract and the standard were measured by the online calculator from AAT Bioquest (https://www.aatbio.com/tools/ic50-calculator). Unpaired

Student's t-test was used to examine the statistical significance. The statistical analyses and graphical presentations were created using the GraphPad Prism software (version 9; GraphPad Software, Inc., San Diego, CA, USA). For all statistical tests, the $p < 0.05$ level was used as the statistical significance criterion.

References

Al-Madhagy, S.A., Gad, S.S., Mostafa, E.S., Angeloni, S., Saad, M.A., Sabry, O.M., Caprioli, G. and El-Hawary, S.S., 2022. A new arsenal of polyphenols to make Parkinson's disease extinct: HPLC-MS/MS profiling, very interesting MAO-B inhibitory activity and antioxidant activity of *Otostegia fruticosa*. *Natural Product Research*, Feb 22:1-6. doi: 10.1080/14786419.2022.2044811.

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Nzekoue FK, Angeloni S, Navarini L, Angeloni C, Freschi M, Hrelia S, Vitali LA, Sagratini G, Vittori S, Caprioli G. 2020. Coffee silverskin extracts: Quantification of 30 bioactive compounds by a new HPLC-MS/MS method and evaluation of their antioxidant and antibacterial activities. *Food Research International*, 133:109128.

Lewandowski, Ł., Bednarz-Misa, I., Kucharska, A.Z., Kubiak, A., Kasprzyk, P., Sozański, T., Przybylska, D., Piórecki, N. and Krzystek-Korpacka, M., 2022. Cornelian Cherry (Cornus mas L.) Extracts Exert Cytotoxicity in Two Selected Melanoma Cell Lines—A Factorial Analysis of Time-Dependent Alterations in Values Obtained with SRB and MTT Assays. *Molecules*, 27(13), p.4193.

Figure **S1.**

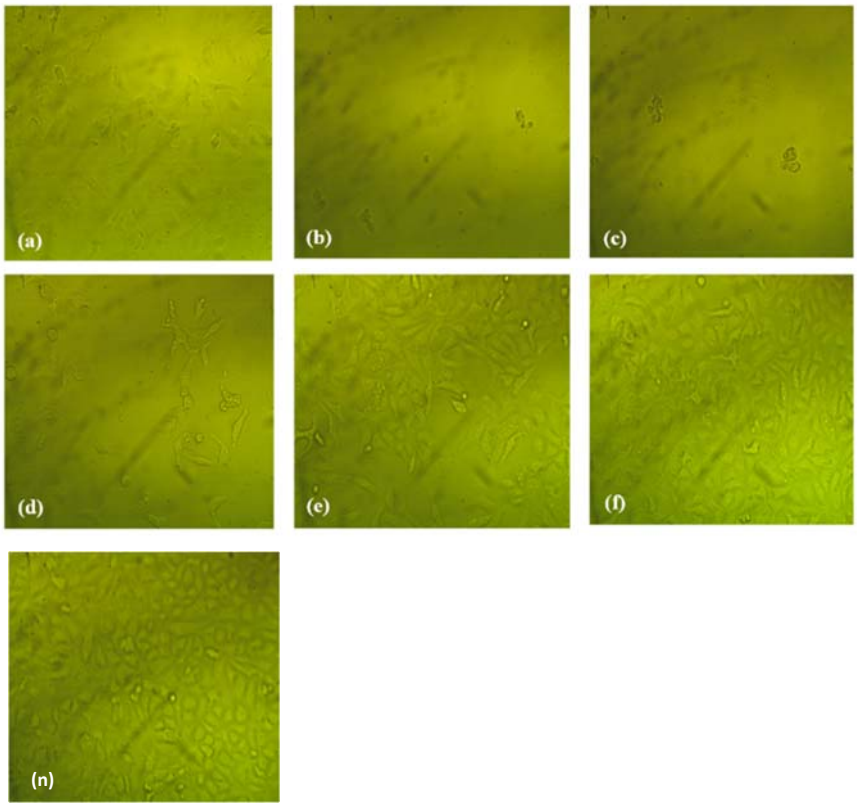


Figure **S2**.

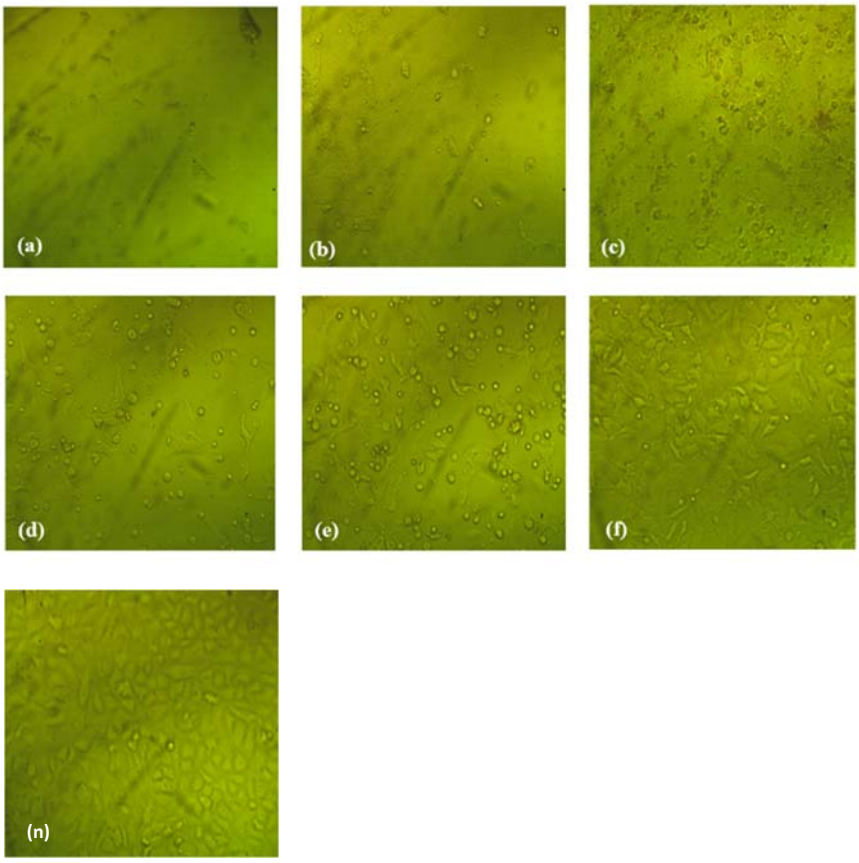


Figure **S3**.

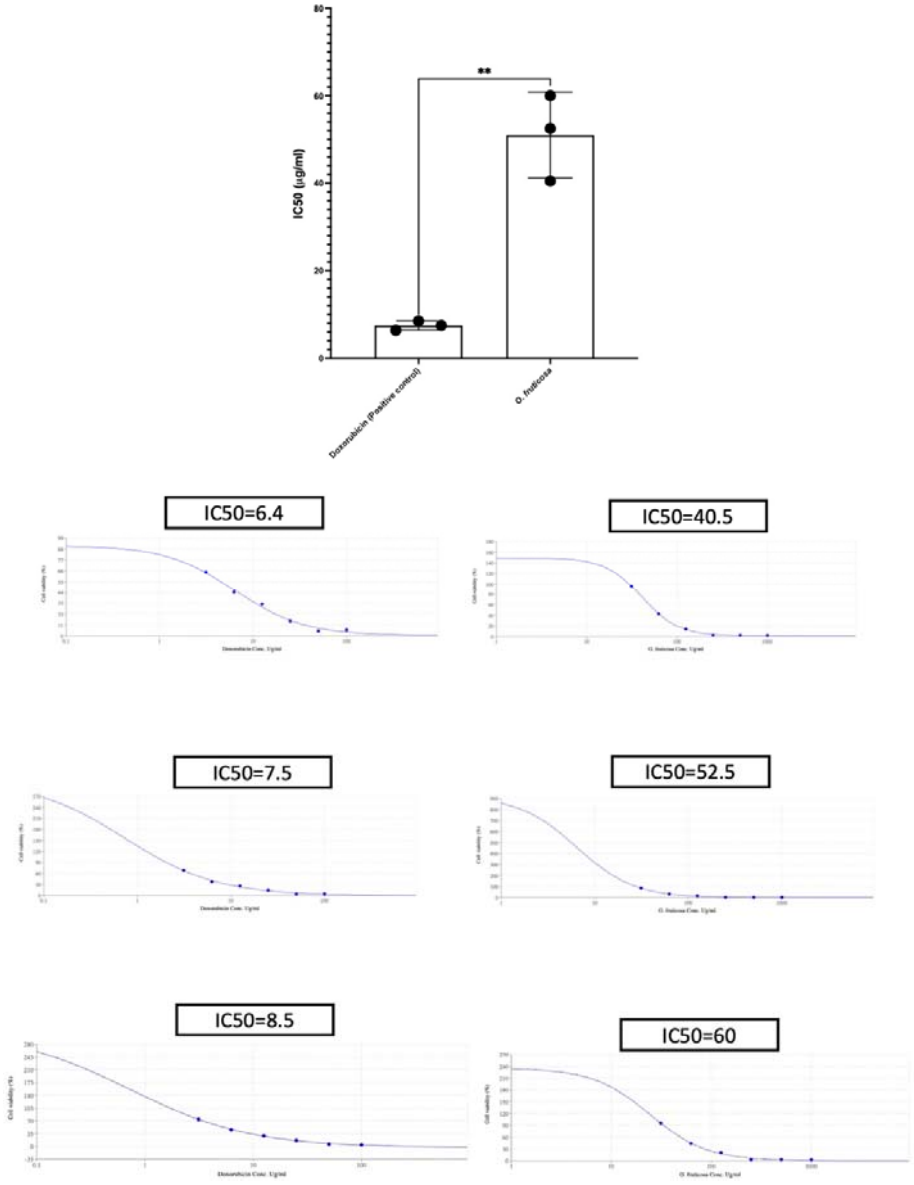

IC50 (µg/ml)
**
Doxorubicin (Positive control)
O. fruticosa
IC50=6.4
IC50=40.5
IC50=7.5
IC50=52.5
IC50=8.5
IC50=60

Figure **S4.**

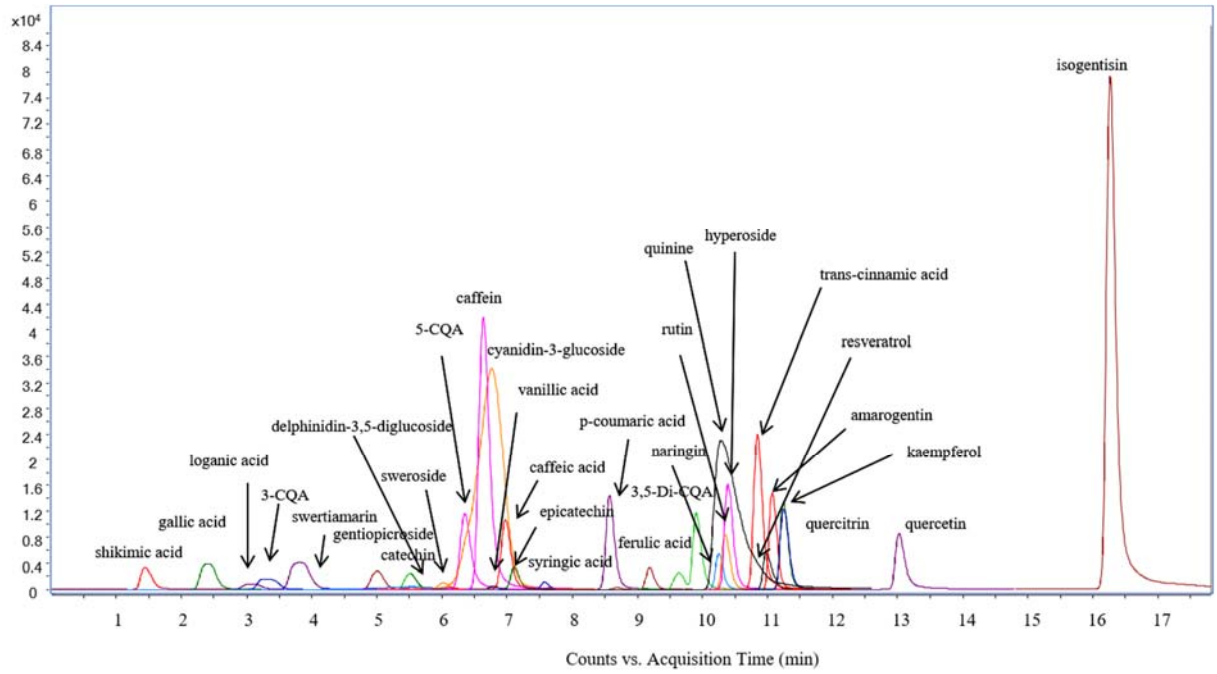
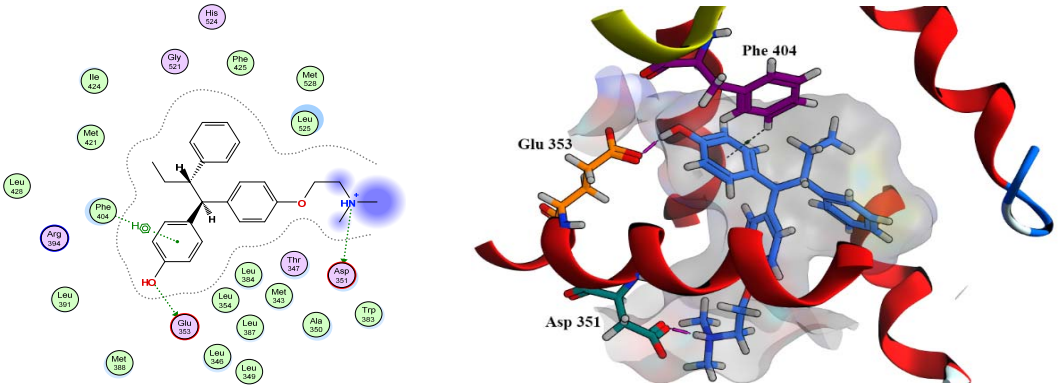

x10⁴
8.4
8
7.4
7.2
6.8
6.4
6
5.6
5.2
4.8
4.4
4
3.6
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Counts vs. Acquisition Time (min)
isogentisin
quinine
hyperoside
trans-cinnamic acid
caffein
5-CQA
rutin
resveratrol
cyanidin-3-glucoside
vanillic acid
amarogentin
delphinidin-3,5-diglucoside
p-coumaric acid
naringin
kaempferol
loganic acid
sweroside
caffeic acid
3,5-Di-CQA
3-CQA
epicatechin
quercitrin
quercetin
swertiamarin
gentiopicroside
ferulic acid
gallic acid
catechin
syringic acid
shikimic acid

Figure **S5.**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Gly 521
Phe 425
Met 528
Ile 424
Leu 525
Met 421
Leu 428
Phe 404
Arg 398
Leu 381
Thr 347
Asp 351
Leu 384
Leu 354
Met 343
Ala 350
Trp 383
Glu 353
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Leu 345
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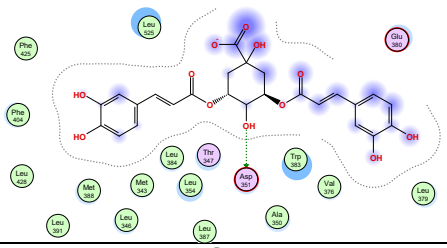
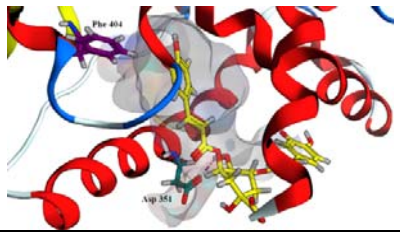
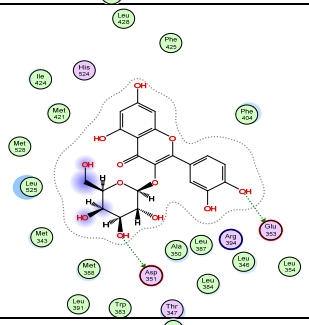
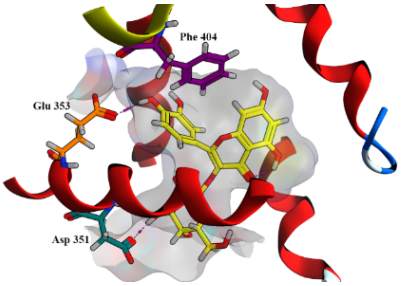
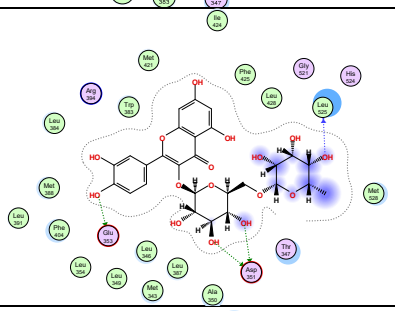
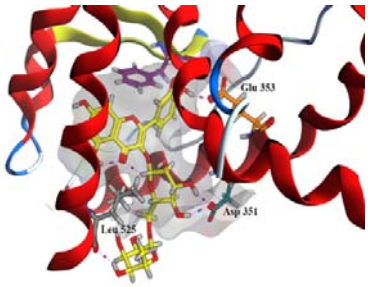
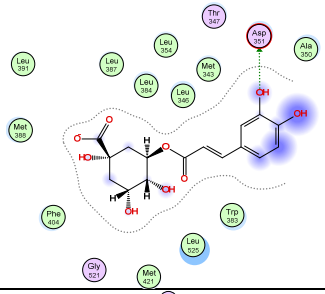
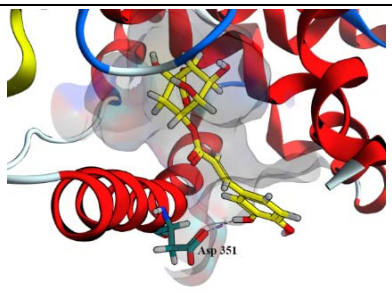
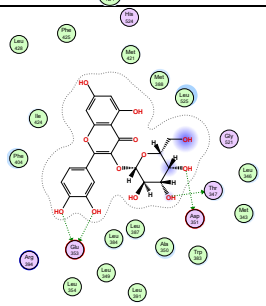
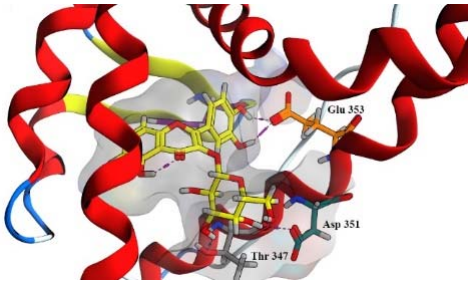
Met 388
Phe 404
Glu 353
Asp 351

Figure **S6.**

(1)		
(2)		
(3)		
(4)		
(5)		

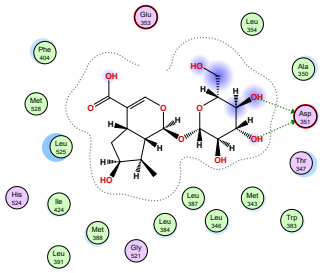
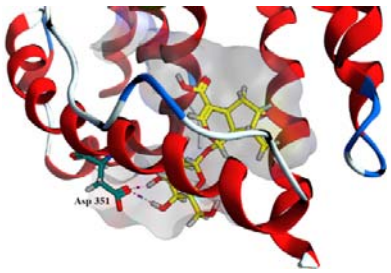
(6)	 Glu 352 Leu 354 Phe 404 Ala 350 Met 326 Asp 351 Leu 329 Thr 347 His 324 Ile 424 Leu 387 Met 343 Met 385 Leu 384 Leu 340 Trp 383 Gly 327 Leu 391	 Asp 351

Table **S1.**

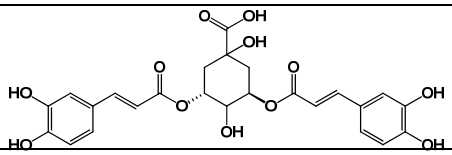
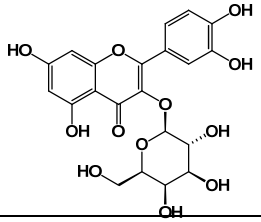
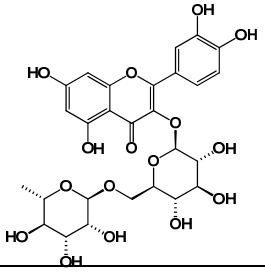
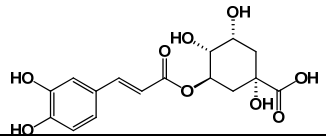
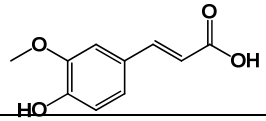
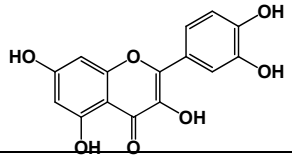
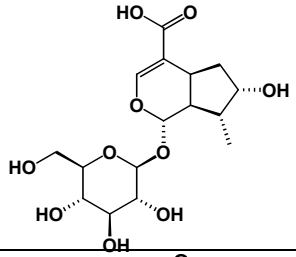
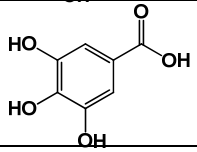
Sample I.D.	**IC_{50}**
Otostegia fruticose methanol leaves extract	IC_{50} = 51 ± 9.836 μg/ml
Doxorubcin	IC_{50} = 7.467 ± 1.05 μg/ml

Table **S2.**

No.	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Polarity	Retention time (Rt) (min)	µg/g
1	Shikimic acid	173	173	Negative	1.40	0.14
			-	-		
2	Gallic acid	169	125[a]	Negative	2.37	1.50
			51			
3	Loganic acid	375	213[a]	Negative	3.13	35.75
			113			
4	3-Caffeoylquinic acid	353	191[a]	Negative	3.58	381.05
			179			
5	Swertiamarin	419	179[a]	Negative	4.89	n.d.
			89			
6	Gentiopicroside	357	177[a]	Positive	5.33	n.d.
			73			
7	(+)-Catechin	289	245[a]	Negative	5.48	n.d.
			109			
8	Delphinidin-3,5-diglucoside	463	300[a]	Negative	5.64	n.d.
			271			
9	Sweroside	403	125[a]	Negative	5.95	n.d.
			179			
10	5-Caffeoylquinic acid	353	191[a]	Negative	6.22	n.d.
			85			
11	Caffeine	195	138[a]	Positive	6.50	231.57
			110			
12	Cyanidin-3-glucoside	449	287[a]	Positive	6.50	n.d.
			403			
13	Vanillic acid	167	108[a]	Negative	6.70	n.d.
			152			
14	Caffeic acid	179	135[a]	Negative	6.87	17.09
			134			
15	(-)-Epicatechin	289	245[a]	Negative	7.03	n.d.
			109			
16	Syringic acid	197	182[a]	Negative	7.48	1.84
			123			
17	*p*-Coumaric acid	163	119[a]	Negative	8.47	3.92
			93			
18	Ferulic acid	193	134[a]	Negative	9.16	2.70
			178			
19	3,5-Dicaffeoylquinic acid	515	353[a]	Negative	9.82	3.87
			191			
20	Quinine	325	79[a]	Positive	10.1	n.d.
			81			
21	Naringin	579	271[a]	Negative	10.17	n.d.
			151			

22	Rutin	609	300[a] 271	Negative	10.34	0.30
23	Hyperoside	463	300[a] 271	Negative	10.43	0.48
24	*Trans*-cinnamic acid	149	131[a] 77	Positive	10.79	0.38
25	Resveratrol	227	185[a] 143	Negative	10.92	n.d.
26	Amarogentin	585	227[a] 245	Negative	11.05	n.d.
27	Kaempferol-3-glucoside	447	284[a] 227	Negative	11.24	n.d.
28	Quercitrin	447	300[a] 301	Negative	11.24	n.d.
29	Quercetin	301	151[a] 179	Negative	13.03	0.08
30	Isogentisin	257	242[a] 214	Negative	16.31	n.d.

Table **S3.**

Compound	Structure	Docking score (kcal/mol)	Binding information	
			Bond type (DH...A)	Distance (Å)
3,5-dicaffeoylquinic acid		-7.5585	ASP 351 (O...H)	2.98
Hyperoside		-6.8387	GLU 353 (O...H) GLU 353 (O...H) THR 347 (O...H) ASP 351 (O...H)	2.99 2.62 2.51 3.12
Rutin		-6.4370	ASP 351 (O…H) ASP 351 (O…H) GLU 353 (O…H) LEU 525 (O…H)	3.26 3.27 2.63 3.00
3-*O*-caffeoylquinic acid		-5.8704	ASP 351 (O…H)	3.13 3.89
Ferulic acid		-5.7761	GLU 353 (O…H) GLU 353 (O…H)	2.98 2.93
Quercetin		-5.7377	GLU 353 (O…H) PHE 404 (6-ring…H)	2.60 3.92
Loganic acid		-5.6373	ASP 351 (O…H) ASP 351 (O…H)	3.07 3.23
Gallic acid		-5.4294	GLU 353 (O…H) GLU 353 (O…H) PHE 404 (6-ring…H)	2.95 3.01 3.91

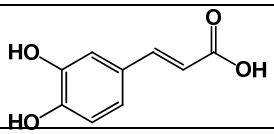
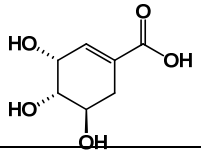
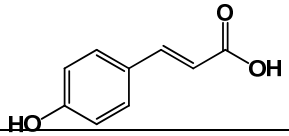
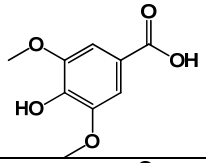
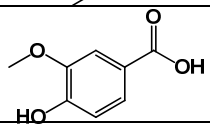
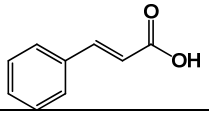
Caffeic acid		-5.3592	GLU 353 (O…H) GLU 353 (O…H) PHE 404 (6-ring…H)	3.30 2.82 3.94
Shikimic acid		-4.9271	GLU 353 (O…H) GLU 353 (O…H)	2.72 3.10
p-Coumaric acid		-4.8064	GLU 353 (O…H) PHE 404 (6-ring…H)	3.90 2.97
Syringic acid		-4.7455	LEU 346 (O…H) PHE 404 (6-ring…H)	2.74 3.99
Vanillic acid		-4.6283	PHE 404 (6-ring…H)	4.30
Trans-Cinnamic acid		-4.2071	ARG 394 (N…H) ARG 394 ionic	3.33 3.33

Figure legends

Figure S1. MCF-7 cell line treated with different concentrations of *Otostegia fruticosa* methanolic extract V.S. untreated cells (magnification power x10) (a) 1000 µg/mL, (b) 500 µg/mL, (c) 250 µg/mL, (d) 125 µg/mL, (e) 62.5µg/mL, (f) 31.25 µg/mL, (n) negative control. The chart shows the toxicity percentage of different concentrations of *Otostegia fruticosa* methanolic extract on MCF-7 cell line

Figure S2. MCF-7 cell line treated with different concentrations of positive control (doxorubicin) V.S. untreated cells (magnification power x10) (a) 1000 µg/mL, (b) 500 µg/mL, (c) 250 µg/mL, (d) 125 µg/mL, (e) 62.5µg/mL, (f) 31.25 µg/mL, (n) negative control. The chart shows the toxicity percentage of different concentrations of positive control (doxorubicin) on MCF-7 cell line.

Figure S3: Determination of the cytotoxic activity of *Otostegia fruticosa* leaves extract using MTT assay as compared to the standard Doxorubicin [t (4) = 7.622, p = 0.0016]. Each vertical-lined bar reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by Unpaired Student's t-test, with the criterion for statistical significance as follows: ****p <0.001.

Figure S4. HPLC-MS/MS chromatogram of a standard mixture of 30 bioactive compounds plotted as overlapped MRM transition of each analyte

Figure S5. 2D and 3D representation of validation experiment of 4-OHT interaction with the X-ray derived structure of ERα+ (PDB code: 2JF9) using MOE software. The best docked pose of 4-hydroxytamoxifen (4-OHT) with estrogen receptor-alpha (ER). Hydrogen bond donor and acceptor interactions are depicted as green dotted arrows in 2D representation and as white dotted line in 3D representation. π- π interaction is indicated as samll green benzen ring and H sign in 2D representation and as blue dotted line in 3D representation. 4-OHT, Phe 404, Asp 351, Glu 353 are colored by blue, cyane, green , and pink, respectively.

Figure S6. 2D and 3D representations of the best poses of hit compounds that resemble antagonist interaction of tamoxifen (left and right sides, respectively). 3,5-O-dicaffeoylquinic acid (1), Hyperoside (2), Rutin (3), 3-O-caffeoylquinic acid (4), Quercitrin (5), Loganic acid (6)

Table Legends

Table S1. Cytotoxicity of *Otostegia fruticosa extract in comparison to standard doxorubicin*

Table S2. Content (μg/g of dried extract) of different bioactive compounds found in the *Otostegia fruticosa* leaves extract.

Table S3. Docking Results of Human Estrogen Receptor with 14 compounds detected by HPLC-MS/MS.