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ORIGINAL ARTICLE

In silico Prediction and Wet Lab Validation of *Arisaema tortuosum* (Wall.) Schott Extracts as Antioxidant and Anti-breast Cancer Source: A Comparative Study

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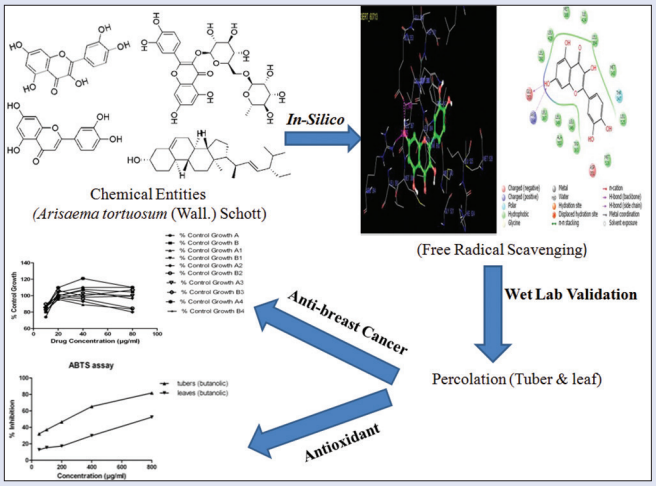
ABSTRACT

Background: Globally, reactive oxygen species have served as an alarm predecessor toward pathogenesis of copious oxidative stress-related diseases. The researchers have turned their attention toward plant-derived herbal goods due to their promising therapeutic applications with minimal side effects. *Arisaema tortuosum* (Wall.) Schott (ATWS) is used in the traditional medicine since ancient years, but scientific assessments are relatively inadequate and need to be unlocked. **Objective:** Our aim was designed to validate the ATWS tuber and leaf extracts as an inhibitor of oxidative stress using computational approach. **Materials and Methods:** The reported chief chemical entities of ATWS were docked using Maestro 9.3 (Schrödinger, LLC, Cambridge, USA) tool and further ATWS extracts (tubers and leaves) were validated with 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ferric-reducing ability of plasma (FRAP), and sulforhodamine B assays experimentally. **Results:** *In silico* results showed notable binding affinity of ATWS phytoconstituents with the receptor (PDB: 3ERT). Experimentally, butanolic tuber fraction confirmed promising antioxidant potential (ABTS: IC_{50}: 271.67 µg/ml; DPPH: IC_{50}: 723.41 µg/ml) with a noteworthy amount of FRAP (195.96 µg/mg), total phenolic content (0.087 µg/mg), and total flavonoid content (7.5 µg/mg) while chloroform fraction (leaves) showed considerable reduction in the cell viability of MCF-7 cell line. **Conclusion:** The current findings may act as a precious tool to further unlock novel potential therapeutic agents against oxidative stress.

Key words: Antioxidant, *Arisaema tortuosum* (Wall.) Schott, *In silico*, MCF-7

SUMMARY

- Quercetin showed top-ranked glide score with notable binding toward 3ERT receptor
- Among extracts, butanolic tubers confirmed as promising antioxidant with remarkable amount of TPC and TFC
- In addition, chloroform fraction (leaves) revealed considerable decline in the cell viability of MCF-7 cell line.



Abbreviations used: ATWS: *Arisaema tortuosum* (Wall.) Schott, DPPH: 2,2′-diphenyl-1-picrylhydrazyl, ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, FRAP: Ferric-reducing ability of plasma, TPC: Total phenolic content, TFC: Total flavonoid content, SRB: Sulforhodamine B

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INTRODUCTION

Cellular exposure to unusual physicochemical or diseased conditions serves as vital means toward the production of free radical species which ultimately directs to oxidative stress. Oxidative stress leads to an alteration in biological systems and further distresses the cell constitution and their applications. Especially, at elevated concentrations, free radicals have reported a link concerning breast cancer.[1,2] Breast cancer is the alarming predecessor of cancer deaths worldwide and the most universal cancer diseases toward women. The pathogenesis has resulted due to hormonal, genetic, and environmental factors. Although there has been a tremendous advancement in the modern medication strategies such as chemotherapy, radiation therapy, hormones, and surgery, successful treatment regarding drug resistance and selectivity remains a big question mark. Previous reports on natural products have indicated their anticarcinogenic and antiproliferative actions against breast cancer cells.[3,4] Therefore, it is quite important to unlock the natural-based agents with selective and nonresistance type potent biological spectrum against oxidative stress diseases.

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Arisaema tortuosum (Wall.) Schott (ATWS); Araceae, commonly known as whipcord cobra lily, is a plant species that is found mainly in the Asian region growing at an elevation of 1500–3000 m. It showed unique purple or green whip-like spadix, approximately 30 cm long, arises from a mouth of flower (Jack-in-the-pulpit).[5,6] Traditionally, the tuber part has valued for antinematodal, anti-inflammatory, and antidote in snake poison, antirheumatic, contraceptive, antihepatotoxic, anticancer, antimicrobial, antioxidant, and esthetic medicinal applications.[7-10] Rhizome (raw/liquid) part is intended for parasitic worms.[11] Fruit is used for the treatment of piles.[12] The leaves in combination with butter, a preparation named "Dardama," is used to treat stomach ache and rheumatism.[13] A chemical literature review on tubers reveals n-alkanes, n-alkanols, phytosterols, alkaloids, fatty acid, amino acids, and flavonoids.[14-17] The plant seed oil has been investigated for insecticidal, anthelmintic, and colic management in animals.[15] The lectin isolated from tubers showed hopeful activity against HT29, SiHa, and OVCAR-5 cell lines.[18] Moreover, the methanolic tuber extract was found in promoting antioxidant, anti-inflammatory, and antiproliferative activities.[17] To date, despite the widespread folk medicine applications of ATWS, there are very few reports in literature which confirm the scientific and therapeutic potential of traditional uses. Moreover, the detailed antioxidant and anticancer investigations on tuber/leaf part with diverse solvents are still very limited. Therefore, the present objectives were designed to computationally predict the ATWS-reported phytoconstituents for probable free radical trapping potential and further confirm its worth on leaf/tuber extracts experimentally as an alternative to synthetic drugs.

MATERIALS AND METHODS

Molecular docking Maestro 9.3 (Schrödinger, LLC, Cambridge, USA) simulations were run at Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India. All extraction solvents utilized were of analytical grade (Rankem and Spectrochem PVT LTD). 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), quercetin, and gallic acid were procured from Sigma-Aldrich (Chemie, Steinheim, Germany). Human breast cancer cell line (MCF-7) study was completed at the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India. Young ATWS leaves and tubers were collected from Bagsiad village (Thunag, Mandi, Himachal Pradesh), India, from August to September 2013, identified by Dr. Sunita Garg, taxonomist, NISCAIR, and voucher specimen was deposited in the herbarium of the CSIR-NISCAIR, New Delhi, India (Ref. No. NISCAIR/RHMD/Consult/2013/2249/30) [Figure 1].

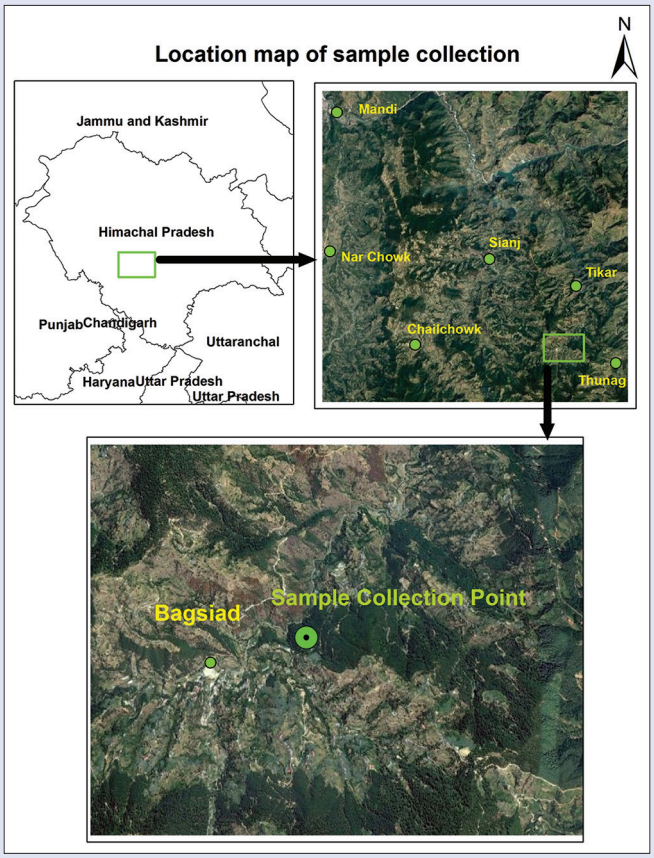

Figure 1: Remote sensing imagery for *Arisaema tortuosum* (Wall.) Schott mapping near Mandi, Himachal Pradesh, India

In silico analysis

Docking was applied on principal reported chemical entities (ATWS) using Maestro 9.3 (Schrödinger, LLC, Cambridge, USA). The estrogen receptor (PDB: 3ERT) was retrieved from online protein data bank and exported into Maestro software. The ATWS ligands were primed with different conformers and docked on 3ERT receptor site for possible free radical scavenging interactions and scoring them to categorize top hit conformation. Molecular docking deals with the ligand interactions such as Van der Waals interactions, H-bonding, and hydrophobic effects with the receptors.[19]

Preparation of extracts and subfractions

The authenticated plant parts were cleaned, shade dried, and grounded to coarse powder properly. The fresh plant materials (leaves/tubers: 250 g) were cold extracted with ethanol:water (95:5, v/v) and dried in a rota-evaporator at 40°C ± 5°C. Furthermore, leaf/tuber crude extract was suspended in water and then partitioned using n-hexane, chloroform, and butanol solvents. Finally, extracts and subfractions were lyophilized and reserved in dark at +4°C awaiting further function.[20]

Antiradical activity

2,2'-diphenyl-1-picrylhydrazyl assay

Radical trapping ability of extracts was calculated as stated in the procedure with slight revision.[21] Aliquots of 1000 µl ethanolic solution containing extracts (0.2–1.0 mg/ml) were added to 2000 µl DPPH (0.1 mM) in ethanol solution. Absorbance was determined after 30 min at 517 nm (Shimadzu 2450, Japan). A graph of percentage inhibition was plotted for each sample. The IC_{50} value, which is the amount of free radical scavenger needed to reduce the original DPPH concentration by 50%, was too intended.

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt assay

ABTS cation radicals were prepared by ABTS decolorization assay.[21] Notably, equimolar ratio measuring 7 mM aqueous solution of ABTS and 2.45 mM $K_2S_2O_8$ was combined and placed in the unilluminated area at room temperature for at least 16 h prior to use, indicating a change in color from blue to green. ABTS radical cation solution was set to absorbance 0.700 ± 0.020 at 724 nm. Aliquots of 50 µl (100–1000 µg/ml) ethanolic solution of extracts were inserted to 2000 µl $ABTS^+$ in the test tubes. The reaction solution was measured after 4 min at 724 nm, and IC_{50} values were estimated (Shimadzu 2450, Japan).

Ferric-reducing ability of plasma assay

Ferric-reducing capacity was calculated as stated by an earlier method with slight amendment.[21] Briefly, 0.3 M acetate buffer (pH 3.6), 10 mM

TPTZ in 40 mM HCL, and 20 mM $FeCl_3$ (10:1:1) were mingled to get fresh ferric-reducing ability of plasma (FRAP) solution. Finally, 0.05 ml of test extract solution was added to 1.5 ml of FRAP reagent and absorbance was note down after 4 min at 593 nm (Shimadzu 2450, Japan).

Total phenolic content

The assay was measured by the Folin–Ciocalteu colorimetric method.[21] An amount of gallic acid calculating 20 µl, 40 µl, 60 µl, 80 µl, and 100 µl was added to 0.5 ml FCP reagent (1N) followed by 1000 µl of Na_2CO_3 (35%), and finally, the volume was made up to 25 ml with distilled water. Briefly, 50 µl of test samples was mixed with the above solution for 35 min at 730 nm. The results were expressed as microgram gallic acid equivalent (µg GAE/mg dry plant extract).

Total flavonoid content

A volume of 0.5 ml of extracts was mixed with 1500 µl ethanol (95%, v/v), 100 µl $AlCl_3$ (10%; w/v) plus 100 µl CH_3COOK (1M) followed by incorporation of 2800 µl distilled water in a 5 ml volumetric flask for 30 min at 415 nm (Shimadzu 2450, Japan). The standard curve was plotted with quercetin by the addition of 500 µl (12.5, 25, 50, 80, and 100 µg/ml) in 80% ethanol.[21]

Determination of *in vitro* anti-breast cancer activity

The anticancer activities of leaf/tuber extracts and their subfractions (10 µg/ml, 20 µg/ml, 40 µg/ml, and 80 µg/ml) were performed at ACTREC, Mumbai, by sulforhodamine B (SRB) assay from earlier reported method. Briefly, RPMI 1640 consisting fetal bovine serum (10%) and 2 mM L-glutamine (2 mM) was employed as cell growth medium. The appropriate cell density aliquots measuring 96 µl/well were inoculated into 96 µl plate for fitting period followed by incubation at 37°C, in 5% CO2, 95% air, and 100% relative humidity for 24 h before adding up of testing laboratory entities.

Hereafter, trichloroacetic acid (TCA) was used as cell-fixing agent for one plate of each cell line which in turn directly correlates with cell line population. The test drugs were dissolved in the proper solvent (400 fold) and frozen before utilized. At the time of drug insertion, an aliquot of frozen concentrate was liquefied and diluted with cell suspension in the required final drug concentrations of 10, 20, 40, and 80 µg/ml, respectively. Adriamycin was indicated as a positive control with comparable above test drug concentrations.

Furthermore, plates were incubated at appropriate conditions for 48 h and assay was ended by adding cold TCA. *In situ* cell fixation was achieved through incorporation of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubating for 60 min at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air-dried. SRB solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered, and the residual dye was detached by washing five times with 1% acetic acid. The plates were air-dried. The bound stain was afterward eluted with 10 mM Trizma® base, and the absorbance was read on an ELISA plate reader at a λ_{max} of 540 nm with 690 nm reference wavelength. The percentage growth was calculated on a plate-by-plate basis for test wells about control wells and expressed as percentage growth = average absorbance of the test or reference well/average absorbance of the control wells × 100.[22]

Statistical analysis

Molecular docking outcome was accounted as glide score with the receptor. The antioxidant results were expressed as IC_{50} values attained from linear regression plots whereas anti-breast cancer data were measured through linear regression method of plots of the cell viability against the log/cm^3 drug concentration of tested compounds.

RESULTS

In silico estimation

Based on the docking results, quercetin has resulted in top glide score for predicting free radical trapping activity against estrogen receptors (PDB: 3ERT). In addition, rutin and luteolin retained second and third rank, respectively, among tested phytoconstituents of ATWS [Table 1 and Figure 2].

Antioxidant assay determination

The antiradical potential of tuber and leaf ATWS extracts was confirmed using the above discussed chemical assays. The antioxidant power of extracts/fractions to counter free radicals was measured up to 1.0 mg/ml and reported regarding percentage inhibition against tested sample concentrations. Moreover, IC_{50} value, namely, the concentration of an antioxidant to trap 50% free radicals was also estimated. The reduction in the IC_{50} value serves as an indicator to greater antioxidant potency. Tables 2 and 3 show our comparative free radical scavenging results using DPPH, ABTS, and FRAP assays. In addition, Table 3 shows a noteworthy amount of total phenolic content (TPC) and total flavonoid content (TFC). Overall, butanolic tuber fraction resulted in significant antioxidant action with ABTS (IC_{50}: 271.67 µg/ml),

Table 1: Free radical scavenging interactions of reported chief phytoconstituents using Maestro software (PDB: 3ERT)

Phytoconstituents	Glide score	Number of H-bonds	H-bond distance (A°)	Amino acid involved
Reference*	−11.3	2	1.82	Gly420
			2.02	Asp351
Quercetin	−8.7	2	1.74	Glu353
			2.24	Arg394
Rutin	−8.6	5	1.92	Glu380
			2.32	Cys530
			2.09	Asp351
Luteolin	−7.8	2	1.84	Thr347
			1.98	Asp351
Stigmasterol	−5.9	1	2.31	Val534
B-sitosterol	−5.9	1	2.47	Val534
Campesterol	−5.5	1	2.39	Val534
Colchicine	−4.6	2	2.34	Leu536
			2.47	Cys530
Cholesterol	−3.7	-	-	-

*Reference: 4-hydroxytamoxifen

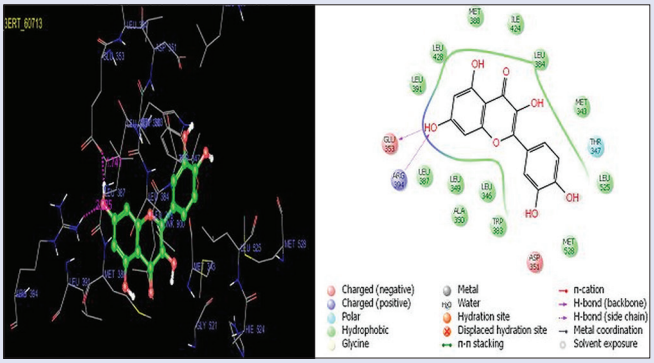

Figure 2: Three- and two-dimensional docking pose view of quercetin with 3ERT receptor

DPPH (IC_{50}: 723.41 µg/ml), FRAP (76.68 µg GAE/mg dry plant extract), TPC (0.087 µg GAE/mg dry plant extract), and TFC (7.5 µg GAE/mg dry plant extract).

Sulforhodamine B assay

The anti-breast cancer effects of ATWS tuber and leaf extracts and their subfractions (ethanolic, n-hexane, chloroform, butanolic, and aqueous) assessed by SRB assay in MCF-7 cell concentration range up to 80 µg/ml. Our SRB results showed that the tuber extracts/fractions up to highest concentration did not cause any momentous decline in the cell viability of MCF-7 cells. Although at 80 µg/ml concentration, cell viability was confirmed as 80.2% in chloroform and 81.2% in n-hexane leaf fractions exposed to MCF-7 cells [Table 4 and Figure 3].

DISCUSSION

In the current scenario, the conviction of natural therapy (plants, phytotherapeutic agents, and phytopharmaceutical products) has resulted in outstanding expansion among consumers due to their safety reasons over synthetic medications.[23] The principal secondary metabolites formed in the plant have shown plentiful tremendous applications against health hazards.[24] Thus, now researchers have turned their awareness toward the natural origin. Previous studies on several species of *Arisaema* have already documented their applications in lore medicine exhibiting febrifuge, antitumor, dermatitis, and anti-inflammatory properties.[25-27] Interestingly, some species have illustrated for their insecticidal, antiepileptic, expectorant, tranquilizer, and cardioprotective actions.[28,29] Despite the well-known ethnomedicinal applications of ATWS, a very few traditional claim activities have been scientifically assessed and explored. Therefore, the present investigations were initially used as *in silico* tools for predicting the possible free radical trapping actions and further confirm its worth on ATWS tuber/leaf extract and its subfractions. Our molecular docking study resulted in top-ranked glide score chemical entities (ATWS) such as quercetin (glide score: −8.7), rutin (glide score: −8.6), and luteolin (glide score: −7.8). The top hit (quercetin) has shown two hydrogen-bonding interactions (Glu353 and Arg394) with breast cancer receptor (PDB: 3ERT). Notably, hydrophobic interactions such as Leu346, Leu347, Leu349, Leu384, Leu391, Leu428, Met388, Ile424, Met343, Leu525, Met528, Trp383, and Ala350 were also observed. These *in silico* studies have provided an important platform to further validate the ATWS experimentally. In our wet laboratory study, comparative leaf/tuber extracts and subfractions were evaluated using antioxidant and anti-breast cancer cell assays. Our findings have revealed that butanolic tuber fraction possesses antiradical potential with a remarkable total phenolic and flavonoid contents whereas chloroform and n-hexane fractions of leaves showed promising cytotoxicity against MCF-7 cell line. However, rest of the ATWS extracts and fractions resulted in very low cytotoxic potential up to 80 µg/ml concentration toward MCF-7 cell line. This study has revealed that computational data showed positive correlation with the experimental information for assessing the free radical scavenging potential.

Table 2: IC_{50} values of ascorbic acid and *Arisaema tortuosum* (Wall.) Schott tuber/leaf extracts in 2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt assays

ATWS (T/L)	IC_{50} value (µg/ml)	
	DPPH	ABTS
Butanolic	723.41/796.44	271.67/775.10
n-hexane	>1000	>1000
Chloroform	>1000	>1000
Aqueous	>1000	>1000
Ethanol	>1000	>1000
Ascorbic acid	9.85	8.43

T/L: Tubers/leaves; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ATWS: *Arisaema tortuosum* (Wall.) Schott; ABTS: 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

Table 3: Relative estimation of ferric-reducing ability of plasma, total polyphenolic contents, and total flavonoid contents in different parts (tubers and leaves) of *Arisaema tortuosum* (Wall.) Schott extracts

Plant extracts[a and b]	FRAP[c]	TPC[c]	TFC[c]
Butanolic	76.68[a] and 42.60[b]	0.087[a] and 0.081[b]	7.5[a] and 5.0[b]

[a and b]Data expressed as [a]tuber and [b]leaf extracts of ATWS, [c]Data expressed as µg of ascorbic acid (FRAP), gallic acid (TPC), and quercetin (TFC) equivalent/mg of ATWS extracts. FRAP: Ferric-reducing ability of plasma; TPC: Total phenolic content; TFC: Total flavonoid content; ATWS: *Arisaema tortuosum* (Wall.) Schott

CONCLUSION

The present findings have revealed that ATWS butanolic tuber fraction showed free radical scavenging action while chloroform and n-hexane fractions of leaves considerably resulted in capable *in vitro* anticancer

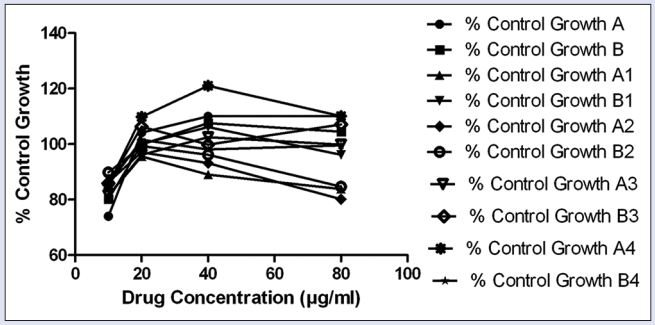

Figure 3: Anti-breast cancer curves of *Arisaema tortuosum* (Wall.) Schott leaf/tuber extracts (A/B: Ethanolic leaf/tuber extract; A1/B1: Hexane leaf/tuber fraction; A2/B2: Chloroform leaf/tuber fraction; A3/B3: Butanolic leaf/tuber fraction; A4/B4: Aqueous leaf/tuber fraction) using sulforhodamine B assay

Table 4: *In vitro* anti-breast cancer results of *Arisaema tortuosum* (Wall.) Schott plant extracts against human breast cancer cell line (MCF-7) by sulforhodamine B assay

Drug concentration (µg/mL)	Percentage control growth									
	A1	A2	A	B	B2	A3	B3	A4	B4	ADR
10	97.1	95.6	>100	>100	>100	>100	>100	>100	>100	−41.4
20	93.0	89.0	>100	>100	>100	>100	>100	>100	>100	−44.6
40	86.3	83.8	>95	>95	>95	>95	>95	>95	>95	−49.6
80	81.2	80.2	>90	>90	>90	>90	>90	>90	>90	−54.4

A/B: Leaf ethanolic extract/tuber ethanolic extract; A1/B1: Leaf hexane extract/tuber hexane extract; A2/B2: Leaf chloroform extract/tuber chloroform extract; A3/B3: Leaf butanolic extract/tuber butanolic extract; A4/B4: Leaf aqueous extract/tuber aqueous extract; ADR: Adverse drug reaction

potential against breast carcinoma (MCF-7) cell. Thus, it could be a potential source of pharmacologically active chemical entities for unlocking novel antioxidant and magic bullets. However, further detailed investigations are required for isolation of the phytoconstituents and understand the mechanistic intracellular pathways accountable for diminishing oxidative stress.

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Conflicts of interest

There are no conflicts of interest.

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