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SYNTHESIS, CHARACTERIZATION AND IN-VITRO ANTICANCER ACTIVITY OF SUBSTITUTED STILBENE DERIVATIVES

Bhavana R.*, Pramila T. and Shebin John

Department of Pharmaceutical Chemistry, Bharathi College of Pharmacy, Rajiv Gandhi University of Health Sciences, Bharathi Nagara, Maddur-571422, Mandya, Karnataka, India.

***Corresponding Author: Bhavana R.**
Department of Pharmaceutical Chemistry, Bharathi College of Pharmacy, Rajiv Gandhi University of Health Sciences, Bharathi Nagara, Maddur-571422, Mandya, Karnataka, India.

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ABSTRACT

In the present work, the novel derivative of substituted stilbene was synthesized from phenylacetic acid and substituted benzaldehyde and screened for their *in-vitro* anti-cancer activity against A375 cell line by MTT assay. The synthesized compounds were characterized by IR, ^{1}H-NMR and Mass analyzer. The results revealed that all synthesized compounds 1a, 2b, 3c, 4d & 5e have a more anti-cancer activity due to presence of nitro and methoxy group.

KEYWORDS: Stilbene, anti-cancer, A375 cell line.

INTRODUCTION

Cancer has become one of the most important health problems characterized by the high cost of treatment and uncertain prognosis. Cancer prevention is currently one of the most widely researched areas. Because there are many mechanisms involved in the pathophysiology of cancer. This situation forces searching for new anticancer agents, of natural origin or chemically synthetized, showing multi-targeting activity and no toxicity to normal cells.

Stilbenes are low molecular weight (~200-300 g/mol), naturally occurring compounds and are found in a wide range of plant sources, used as aromatherapy products, and dietary supplements.

Stilbenes exist as stereoisomers in *E* and *Z* forms, depending on where functional groups are attached in relation to one another on either side of the double bond. Naturally occurring stilbenes overwhelmingly exist in the *Z* form. It has been postulated and scientifically verified that the *E* and *Z* forms of stilbenes elicit different pharmacological activities. Research has revealed that the *Z* form exhibits more potent activity compared to the *E* form across various anti-cancer and antioxidant assays.[1]

There is a growing interest in stilbene derivatives because many activities have been observed in some of the naturally occurring as well as the synthetic stilbenes. Modification in their structure has offered a high degree of diversity that has proved useful for the development of new therapeutic agents with improved potency and lower toxicity. Activities include antimicrobial,[2,3,4] antioxidant[4,5,6] anticancer,[4] antileukemic,[7] anti-platelet aggregative,[8,9] protein tyrosine kinase inhibitory,[10] anti-inflammatory,[11] anti-HIV,[12] anti-herpes simplex virus,[13] Alzheimer's disease[14] and antidiabetic.[11,15] The pharmacological profits of employing the stilbene nucleus are due to the fact that this structure acts as a key pharmacophore for the biological activity.

MATERIALS AND METHODS

Identification and Characterization

The synthesized compounds were scaled for yield and purified by recrystallization with suitable solvent system.

Analytical techniques

Melting point determination: The melting point of the synthesized compounds was determined in open capillary tube and recorded in °C without correction. It is widely used physical constant in the characterization of an organic compound.

Thin layer chromatography (TLC): Thin layer chromatography was performed on precoated silica gel plates (604 GF 254 Merck) with suitable solvent system. This method is also applied to determine the progress of the reaction and to identify the purity of the synthesized product.

Instrumentation: The techniques employed for the characterization of the synthesized compounds were IR spectra, ^{1}H-NMR, Mass spectra and elemental analysis.

IR spectra (Fourier transform IR Spectrometer model Shimadzu using KBr pellet), ^{1}HNMR (DMSO-$d_6$in Amx-400 MHz liquid state NMR spectrometer) Chemical shifts(δ) are reported in parts per million downfield from internal reference Tetramethyl Silane (TS), VIT, Vellore, LC-MS spectrophotometer, SAPALA Organic Pvt. Ltd., Chennai.

Chemicals and Reagents: phenyl acetic acid, benzaldehyde, 2-nitrobenzaldehyde, 3-nitrobenzaldehyde, 4-methoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, tri ethylamine, acetic anhydride, thionyl chloride, benzene, hexane, ethyl acetate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), 1-hydroxybenzotrizole (HOBT), dimethylformamide (DMF), Ethanol, methanol and benzylamine.

Scheme of synthesis

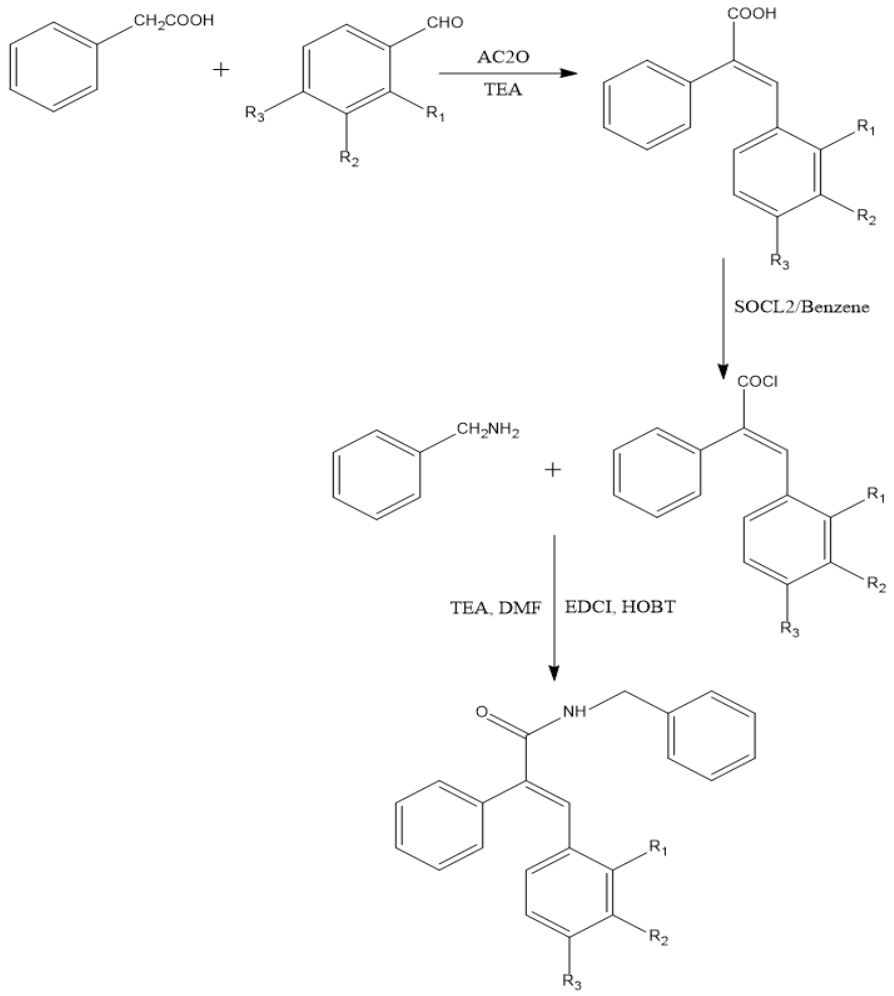

Table 1: Different types of substitution.

SL. NO.	R_1	R_2	R_3
1	-H	-H	-H
2	$-NO_2$	-H	-H
3	-H	$-NO_2$	-H
4	-H	-H	$-OCH_3$
5	-H	$-OCH_3$	$-OCH_3$

Step 1: Preparation of stilbene acid

Phenyl acetic acid (0.029mol), substituted benzaldehyde (0.02mol) and triethylanime (0.02mol) in acetic anhydride (10ml) was taken in a round bottomed flask. The reaction mixture was heated gently and refluxed for 30 minutes and cool to 90^{0}C. The reaction mixture poured into crushed ice slowly and cool to 15-20^{0}C for 1 hour. The crude product was recrystallized from ethyl acetate.

Step 2: Preparation of stilbene chloride

An equimolar quantity of Stilbene acid and thionyl chloride in benzene (10ml) was refluxed for 6 hours. The excess thionyl chloride and benzene were removed and residue was kept under vacuum for 30 minutes.

Step 3: Preparation of substituted stilbene amide
Stilbene chloride (1mmol) was dissolved in dimethyl formamide (10ml) into this TEA(3mmol) was added and stirred for 15minutes. EDCI (1.2mmol), HOBT (1mmol) and benzylamine (1.2mmol) was added to the reaction mixture and stirred at room temperature for overnight. The reaction mixture was poured into ice cold water and the resulting precipitate was filtered and washed with water. The residue was dried under vacuum and stored in desiccators.

***In-vitro* cytotoxicity activity**
Preparation of test solutions
For MTT assay, serial two-fold dilutions (6.25 – 100 µg) were prepared from this assay.

Cell lines and culture medium
A375 cell line was procured from NCCS, stock cell was cultured in medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37^{o}C until confluent.

Procedure
The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µL of the diluted cell suspension (1 x 10^4cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µL of different concentrations of test samples were added on to the partial monolayer in microtiter plates. The plate was then incubated at 37^{o}C for 24 h in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 20µL of MTT (2 mg/1 mL of MTT in PBS) was added to each well. The plate was incubated for 4h at 37^{o}C in 5% CO_2 atmosphere. The supernatant was removed and 100µL of DMSO was added and the plate was gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570nm. The percentage of viability was calculated using the following formula,% viability = Sample abs/Control absx 100.

RESULT AND DISCUSSION

Physical characterization and spectral data
(2*E*)-*N*-benzyl-2,3-diphenylprop-2-enamide (1a) is a White crystal; Molecular weight-313.39236; Solubility-Ethanol, DMSO; Melting point-195-198°C; Percentage yield-80%; TLC solvent system- Hexane:ethyl acetate (7:3); R_f value- 0.57; Composition- C(84.31%) H(6.11%) N(4.47%) O(5.11%); IR (ν cm−1): 3288.85 (NH Stretching of NH of CONH group broad peak H bonded NH); 3088 (ArCH stretch); 2962.50 & 2860.59 (CH stretch of CH_2 both symmetric and asymmetric); 1675 (C=O of CONH); 1612 (CH=C stretching); 1612.38 & 1529.78 (C=C ring stretch); 1428.82 & 1372.62 (CH bending of CH_2 both symmetric and asymmetric); 1428.82 (CN Stretch); 1270.42 (CO Stretch); 712.03 (Mono substituted benzene).

^{1}H-NMR (DMSO) $\delta\delta$ ppm: δ4.74 (2H, s, 2H of CH_2); δ7.15 (H, s, s of CH=C); δ7.33 to 9.33 (15H, m, ArH).

Mass (ESI-MS): Molecular weight 313, molecular ion peak is observed as M+1 at m/e value 314 which agrees with the Molecular weight of the compound. This confirms the structure.

(2*E*)-*N*-benzyl-3-(2-nitrophenyl)-2-phenylprop-2-enamide (2b) is a Yellow crystal; Molecular weight-358.38992; Solubility-Ethanol, DMSO; Melting point-223-225°C; Percentage yield-62%; TLC solvent system-Hexane:ethyl acetate (7:3); R_f value-0.52; Composition-C(73.73%) H(5.06%) N(7.82%) O(13.39%); IR (ν cm−1): 3267.27 (NH Stretching of NH of CONH group broad peak H bonded NH); 3079.37 (ArCH Stretching); 2935.96 & 2867 (CH Stretching of CH_2); 1675 (C=O of CONH); 1633.76 (CH=C Stretching); 1633.76 & 1574.05 (C=C ring Stretching); 1435 (CN Stretching); 1271 (CO stretching); 710 (ortho substituted benzene); 737 (mono substituted benzene).

^{1}H-NMR (DMSO) $\delta\delta$ ppm: δ4.76 (2H, s, CH_2); δ7.26 to 9.32 (15H, m, 14ArH & H of CH=C); δ12.74 (H, 6s, H of NH-C=o).

Mass (ESI-MS): Molecular ion peak is M+1 appeared at m/e at 359 since the molecular weight of the compound agree with the M+1 ion peak confirms the structure of the compound.

(2*E*)-*N*-benzyl-3-(3-nitrophenyl)-2-phenylprop-2-enamide (3c) is a Yellow crystal; Molecular weight-358.38992; Solubility-Ethanol, DMSO; Melting point-182-187°C; Percentage yield-55%; TLC solvent system-Hexane:ethyl acetate (7:3); R_f value-0.6; Composition-C(73.73%) H(5.06%) N(7.82%) O(13.39%); IR (ν cm−1): 3299.19 (NH Stretching of NH of CONH group broad peak H bonded NH); 3091.56 & 3050.37 (ArCH Stretching); 2937.18 & 2866.12 (CH Stretching of CH_2 group); 1688.21 >C=O of NH group); 1599.51, 1533.33 & 1502.42 (C=C ring Stretching); 1599.51 (C=C Stretching); 1533.53 & 1378.73 (NO_2 Stretching); 1432.06 & 1378.73 (CH bending of CH_2 group); 1239.23 (CO Stretching); 1378.73 (CN Stretching); 746.23 & 688.92 substituted benzenes).

Mass (ESI-MS): Molecular weight is 358. Molecular ion peak is M+1 appeared at m/e 359. This agree with the Molecular weight of the compound hence, confirms the structure of the compound.

(2*E*)-*N*-benzyl-3-(4-methoxyphenyl)-2-phenylprop-2-enamide (4d) is a White crystal; Molecular weight-343.41834; Solubility-Ethanol, DMSO; Melting point-170-175°C; Percentage yield-73%; TLC solvent system-Hexane:ethyl acetate (7:3); R_f value-0.48; Composition-

C(80.44%) H(6.16%) N(4.08%) O(9.32%) IR (ν cm−1): 3274.08 (NH Stretching of NH of CONH group broad peak H bonded NH); 3047.41 (ArCH Stretching); 2945.64 (CH Stretching of CH_3); 2850.87 (methoxy & CH2 group); 1684.42 (C=O Stretching of CONH); 1607.04 (CH=C< Stretching); 1607.04, 1538.46 & 1511.53 (C=C ring Stretching); 1444.14 & 1340 (CH bending of CH_3 of OCH_3 and CH2 group); 1444 (C-N Stretching); 1277.59 (CO Stretching); 1007.27 (C-O-C Stretching); 826.49 (1,4 disubstituted benzene ring); 760.71 (Mono substituted benzene ring).

Mass (ESI-MS): Molecular weight is 343. Molecular ion peak obtained as M+1 & appeared at m/e 344. This agree with the Molecular weight of the compound, hence, confirms the structure of the compound.

(*E*)-*N*-benzyl-3-(3,4-dimethoxyphenyl)-2-phenylacrylamide (5e) is a White crystal; Molecular weight- 373.44432; Solubility-Ethanol, DMSO; Melting point- 178-181°C; Percentage yield- 65%; TLC solvent system- Hexane:ethyl acetate (7:3); R_f value-0.65; Composition- C(77.19%) H(6.21%) N(3.75%) O(12.85%) IR (ν cm−1): 3288 (NH Stretching of NH of CONH group broad peak H bonded NH); 3094.19 (ArCH Stretching); 2959 & 2869 (methoxy & CH2 group); 1686.32 (C=O of CONH group); 1605 (CH=C< Stretching); 1605 & 1504 (C=C ring Stretching); 1438 & 1340 (CH bending of OCH3 & CH2 group); 1266 (CO Stretching); 1001.58 (C-O-C Stretching & methoxy) 877.78 & 723.12 (Tri-substituted and Mono-substituted benzene).

^{1}H-NMR (DMSO) $\delta\delta$ ppm: δ3.8 (NH, s, 2methoxy group); δ4.49 (2H, s, CH2); δ7.01 (H, s, H of CH=C); δ7.30 to 9.32 (13H, m, ArH); δ12.49 (H, s, H of NH of CONH).

Mass (ESI-MS): Molecular ion peak appeared as M+1 at m/e 374. Molecular weight value agrees with Molecular ion peak hence confirm a structure of a compound.

***In-vitro* cytotoxicity activity**

From the *in-vitro* cytotoxicity studies, by using MTT assay on A375 cell line the obtained data revealed that all the synthesized compounds proved that having less toxicity compared to standard.

Newly line cells by MTT assay and results are depicted in table 2 for synthesized compounds were tested in a concentration range from 6.25 to 100 µg/ml on A375 cell line cells for 48 hrs by dye exclusion method. The synthesized Stilbenes were found to possess cytotoxicity on human synthesized Stilbene derivatives were evaluated for their cytotoxicity against A375 cell cancer cell lines in dose dependent manner. Growth curves showed that the complexes displayed inhibition effect and the IC50. The IC50 Value of the given test samples 1a, 2b, 3c, 4d, 5e and standard (Cisplatin B) is 59.21, >100, 44.03, 70.20, >100 and 2.95 µg/ml, respectively in A375 cell line.

Table 2: Cytotoxicity activity of substituted Stilbene derivatives against A374 cell line.

SL. NO	Conc (µg/ml)	Compound 1a	Compound 2b	Compound 3c	Compound 4d	Compound 5e	Cisplatin
1	6.25	87.28785	78.88182	82.06874	81.11885	79.22126	47.99969
2	12.5	77.69597	76.00095	69.35604	75.05174	75.28873	28.94978
3	25	73.93268	74.74542	58.78059	68.71302	67.15247	22.16922
4	50	60.5079	61.69485	54.13502	61.89825	65.0517	16.74658
5	100	20.37161	58.84793	32.47421	35.5931	60.57668	15.59413

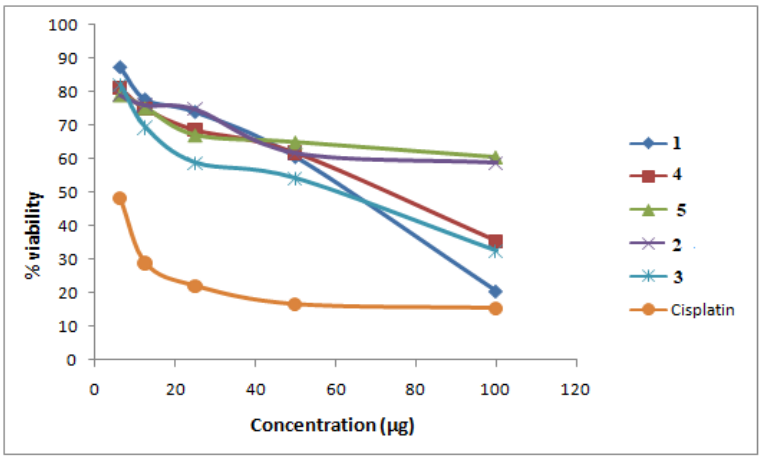

Fig. 1: Graphical presentation of *in-vitro* cytotoxic effect of Stilbene derivative against A375 Cell Line.

The cytotoxicity screening by MTT assay indicated that stilbenes showed more cytotoxicity toward the human cancer cell line A375. The effects of stilbenes on the viability of A375 cells were evaluated after 48 hrs. These

studies have clearly shown that the inhibition of cell growth was dose dependent manner. The reduction of cell growth in the presence of stilbenes cloud be due to either apoptosis or necrosis.

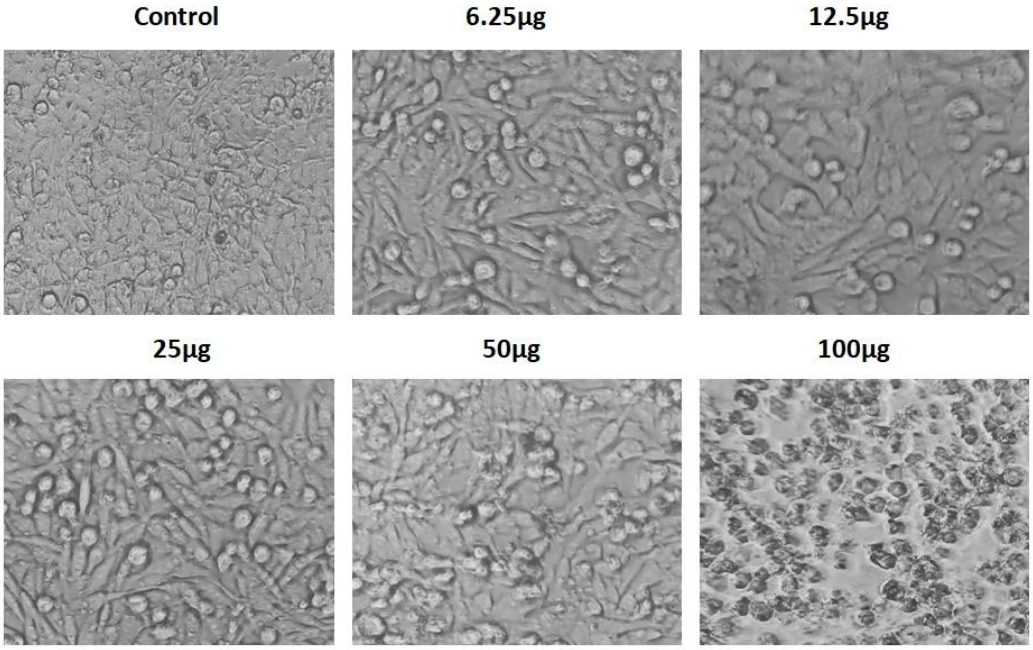

Fig. 2: *In-vitro* cytotoxicity studies of compound 1a against A375 cell lines of various concentration.

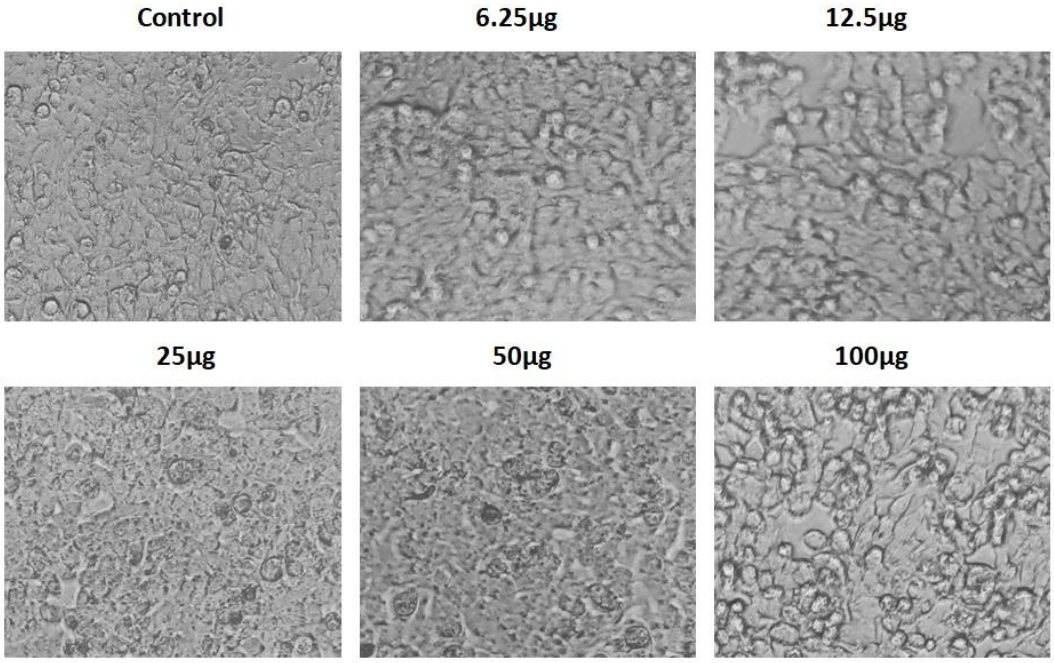

Fig. 3: *In-vitro* cytotoxicity studies of compound 2b against A375 cell lines of various concentrations.

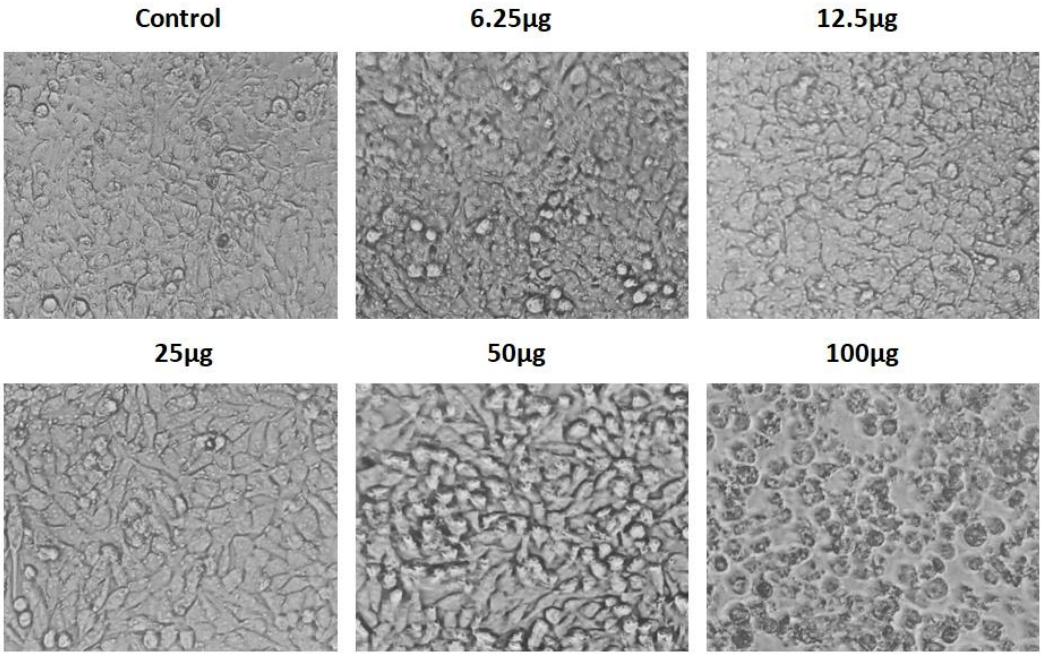

Fig. 4: *In-vitro* cytotoxicity studies of compound 3c against A375 cell lines of various concentrations.

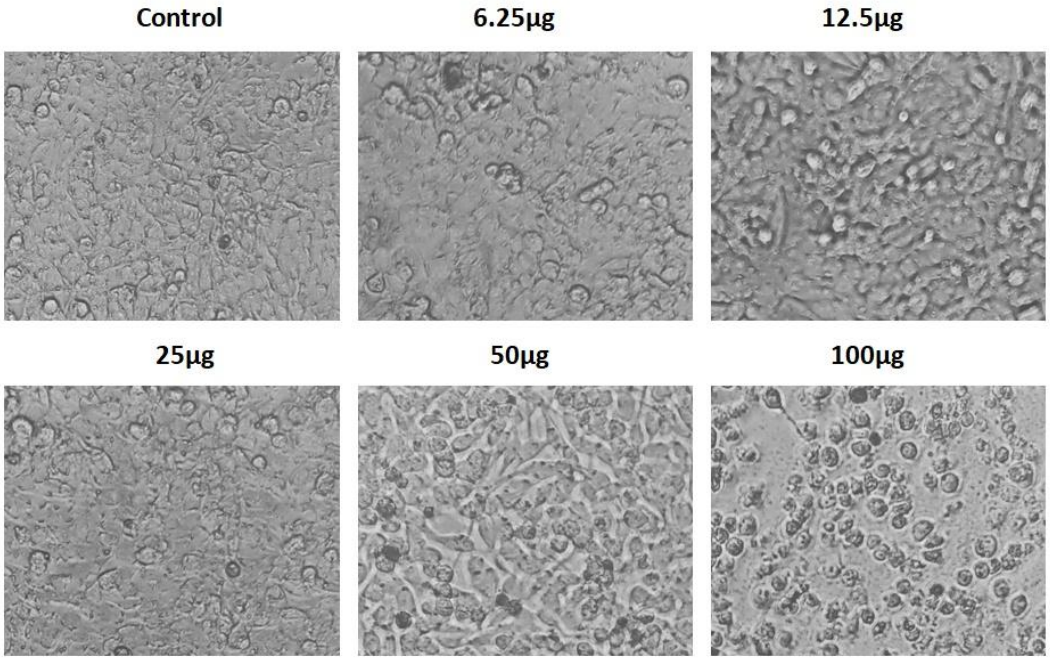

Fig. 5: *In-vitro* cytotoxicity studies of compound 4d against A375 cell lines of various concentrations.

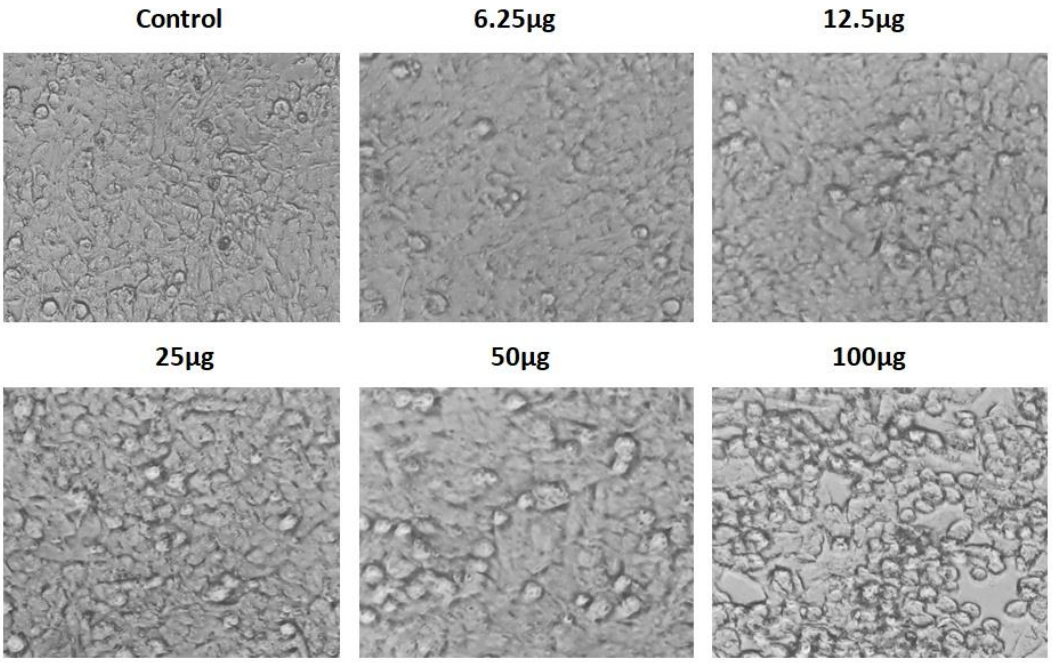

Fig. 6: *In-vitro* cytotoxicity studies of compound 5e against A375 cell lines of various concentrations.

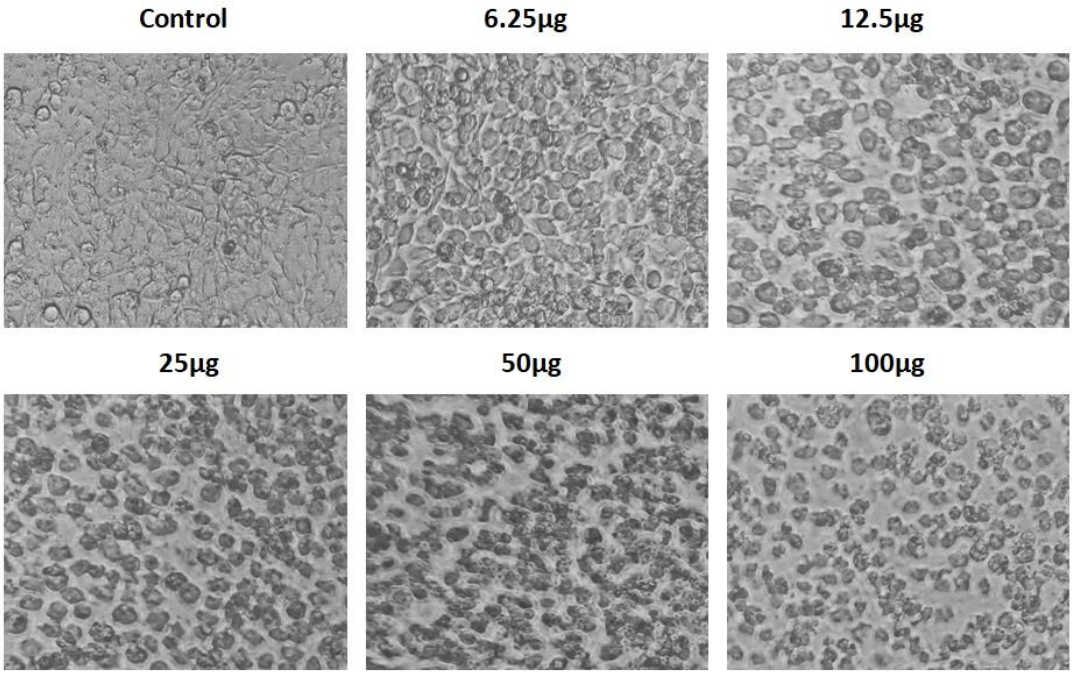

Fig. 7: *In-vitro* cytotoxicity studies of standard against A375 cell lines of various concentrations.

CONCLUSION

Stilbenes belong to the important classes of natural Phytoalexins. These compounds possess versatile type of biological activities; anti-microbial, anti-oxidant, anti-cancer, anti-leukemic, anti-platelet aggregative, protein tyrosine kinase inhibitory, anti-inflammatory, anti-HIV, anti-herpes simplex virus, Alzheimer's disease and antidiabetic. A series (2*E*)-*N*-benzyl-2,3-diphenylprop-2-enamide derivatives (1a-5e) have been synthesized from phenylacetic acid with aryl aldehyde in the presence of TEA and acetic anhydride and obtained stilbenic acid coupled with phenyl amine molecule. The yield of all synthesized compounds was found to be in the range of 55-80%. Structure of newly synthesized compound were confirmed and characterized by physical data like melting point, TLC and analytical data such as IR, 'H-NMR and mass spectra. The synthesized compounds were screened for their *in-vitro* cytotoxicity activity. From the *in-vitro* cytotoxicity studies, by using MTT assay on A375 cell line the obtained data revealed that

all the synthesized compounds proved that having less toxicity compared to standard. The cytotoxicity screening by MTT assay indicated that all the synthesized compounds (1a-5e) showed more cytotoxicity toward the human cancer cell lines such as A375. These studies have clearly shown that the inhibition of cell growth was in dose-dependent manner and the reduction of cell growth in the presence of synthesized stilbenes could be due to either apoptosis or necrosis.

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