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ANTI-CANCER POTENTIAL OF *MORUS INDICA* HYBRID VARIETIES IN HT-29 CANCER CELL LINES: AN EXPLORATORY STUDY

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ABSTRACT: Complementary alternative medicine focuses on discovering the potential of plants in prevention and management of chronic diseases. Although *Morus* species has been screened for various biological activities, studies reporting the pharmacological effects of *Morus indica* (MI) hybrid varieties *viz*., V1 and S36 is scarce. This study focused on screening the phytochemical composition of *Morus indica* V1 (MV1) and *Morus indica* S36 (MS36) and evaluate its antioxidant and anti-cancer potential. The qualitative analyses revealed the presence of all major phytochemicals except alkaloids. The results of the quantitative analyses showed a better phytochemical profile in MV1 than MS36 and the values differed significantly ($p<0.05$). The antioxidant activity was higher in methanol extracts of both the MI varieties followed by ethanol and aqueous extracts since the extractability of phytochemicals are higher in organic solvents. MV1 had a better phytochemical profile compared to MS36 and therefore was selected for screening the *in-vitro* anti-cancer properties. Among the methanol and aqueous extract of MV1, aqueous extract had the lowest IC_{50} value *viz*., 55 µg/mL. The results of apoptotic activity of aqueous extract of MI at a dosage of 160 µg/mL revealed induction of late apoptosis and higher cell deathin HT-29 cell lines than the standard drug colchicine at 20µM concentration. Results indicate the potential of *Morus indica* hybrid variety MV1 as a source for nutraceutical compounds with anti-cancer properties. The study paves a way for further screening and isolation of compounds with anti-cancer and other vital biological activities.

INTRODUCTION: Phytochemicals are biologically active, non-nutritive and naturally occurring chemical compounds found in plants which provide health benefits to humans. Sources of phytochemicals include leaves, roots, fruits, seeds, bark of medicinal plants and dietary sources such as vegetables, legumes, whole grains, nuts, fungi, herbs and spices.

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Phytochemicals present majorly in these sources include phenolic compounds, terpenoids, tocopherols, carotenoids, phytosterols, alkaloids *etc*. Though present in smaller amounts, secondary plant metabolites from these sources possess protective biological roles which include antioxidant, antimicrobial, anti-cancer, anti-inflammatory and immune enhancing effects and therefore can be used in preventing and managing an array of degenerative diseases [1].

The science of reverse pharmacological approach and complementary alternative medicine focuses on discovering the potential of plants in prevention and management of chronic diseases. Several *in-vitro* and *in-vivo* model systems are used for

screening the biological activities of phytochemicals. The antioxidant activity of plant extracts can be assayed using methods *viz*., 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) assay, ABTS radical scavenging assay and lipid peroxidation assay. Methods that confirm the presence of anti-cancer potential *in-vitro* include tryphan blue dye exclusion assay, Lactic dehydrogenase activity, [3-(4, 5 dimethylthiazol-yl)-2, 5- diphenyltetrazolium bromide] (MTT) assay, (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]- 2H tetrazolium- 5-carboxy-anilide inner salt) (XTT) assay, Sulforhodamine B (SRB) assay and *in-vivo* method such as Ehrlich ascites carcinoma tumor model in mice.

Morus indica (MI), commonly known as mulberry, cultivated both under irrigated and rain fed conditions, has special significance in sericulture industry as it serves as the primary food plant for the monophagous insect *Bombyx mori*. Due to its chemical composition and pharmacological functions it is being utilized as a medicinal plant.

Many active compounds have been isolated from different parts of mulberry plant *viz*., moranolin, deoxynojirimycin, moran (glycopeptides), alba-furan, albanol, morusin, kuwanol, hydroxymoricin, hydrophobic flavonoids (flavones and flavonone), 2-arylbenzofuran, polyphenols, carotenoids, vitamins A, C, E; ethylacetate, γ -aminobutyricacid, flavanicsetc, which exhibit biological activities such as hypoglycemic, hypolipidemic, anti-oxidative, anti-inflammatory, anti-allergic, vaso-active and neuro-protective effects [2].

The antioxidant [3, 4], hypoglycemic [5, 6] and hypo-cholesterolemic [7] effects of MI have been evaluated in our laboratory using various model systems. The anti-cancer effect of *M. alba* and *M. nigra* in cancer cell lines are well documented in literature [8-10].

Though there are various studies reported on anti-cancer potential of genus Morus, there are no studies which report the anti-cancer activity of hybrid varieties *viz*., *Morus indica* V1 (MV1) and *Morus indica* S36 (MS36) on HT-29 colon cancer cell lines. Hence, the present study was planned to screen the phytochemical composition and evaluate the antioxidant and anti-cancer potential of MI hybrid varieties.

MATERIALS AND METHODS:

Chemicals: Chemicals such as DPPH, MTT, L-glutamine penicillin streptomycin solution; 2,7-dichlorofluorescin diacetate, triton X-100, rutin, rhein and colchicine were purchased from Sigma Aldrich chemicals, USA. Propidium iodide, trypsin-EDTA solution, trypan blue dye, RNase A solution, Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate-buffered saline (PBS), atropine and linalool were purchased from Himedia chemicals, India. Annexin V-FITC apoptosis kit was purchased from Invitrogen, USA. All other chemicals used were of analytical grade

Cancer Cell Lines Procurement and Culturing: The anti-cancer assays were carried out at Skanda Lifesciences Pvt. Ltd, Bengaluru, Karnataka, India. Colon cancer cell line (HT-29) was procured from American Type Culture Collection (ATCC), sub-cultured and maintained at optimum conditions.

The cells were maintained in DMEM medium containing 10% FBS, 1mM sodium-bi-carbonate, glucose and L-glutamine penicillin streptomycin solution at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was replaced twice in a week. Confluent cells (70-80%) were trypsinized and plated in 6 and 96 well plates for the experiments

Collection and Processing of Plant Samples: Two hybrid varieties of MI *i.e.*, MV1 and MS36 were selected for the study. The samples were identified by Dr. Basavaiah, Professor, DOS in Sericulture, University of Mysore, Mysuru and procured from the Sericulture department of the University. The voucher specimen was retained in the laboratory for further reference. The leaves were cleaned, washed and dried in a hot air oven at 40 °C for 2-3 days. The dried leaves were powdered finely using a mixer and stored in air tight container until further use.

Preparation of Extracts: About 15g of powdered leaves sample was dissolved in 100ml of the respective solvents (ethanol, methanol and water) to perform screening of phytochemicals and antioxidant assays. The samples were extracted for 6 h in a mechanical shaker. The samples were centrifuged for 10min at 3500 rpm, then filtered

using Whatman no. 40. The filtrate of ethanol and methanol samples of MI were evaporated at 40 °C in a vacuum oven, while the filtrate of aqueous samples were lyophilized in a Modulyo D Freeze Dryer, Thermo Electron Corporation, United States. The extracts were stored in vials at 4°C until further use. The final yield of each of the extracts of the samples was calculated.

A stock solution of the extracts for the anti-cancer assays were prepared by dissolving in dimethyl-sulfoxide at a concentration of 32 mg/mL and serially diluted to different concentrations of the extract *viz*., 320, 160, 80, 40, 20 and 10µg/mL in DMEM complete media.

Qualitative Analysis of Phytochemicals: The qualitative screening of phytochemicals in MI varieties was conducted in dried sample for few of the phytochemicals and in extracts as followed in the standard protocol. Tannins, flavonoids, polyphenols, phytosterols and glycosides were analyzed in the extracts whereas saponins, terpenoids and alkaloids were analyzed on dry basis.

Tannins were detected by addition of 1% lead acetate [11]; flavonoids [12] and polyphenols by addition of 5% ferric chloride [11]; phytosterols by Salkowski's test [11], glycosides by Keller Kiliani test [13], saponins by foam test [11], terpenoids by the addition of chloroform and sulphuric acid [12]; and alkaloids by the addition of picric acid [12].

Quantitative Analysis of Phytochemicals: The extracts of both the MI hybrid varieties were subjected to quantitative screening of phyto-chemicals. Polyphenols, glutathione, tannins, saponins, cardiac glycosides and anthraquinone glycosides were estimated on dry basis; flavonoids, terpenoids and alkaloids in the extracts; and ascorbic acid on fresh basis.

The total phenolic content of the samples was estimated according to the Folin-Ciocalteu method and results were expressed as gallic acid equivalents per g [14]. Reduced glutathione (GSH) was estimated based on the reaction of 5,5'-dithio(bis) nitrobenzoic acid (DTNB) with compounds containing sulphydryl groups. GSH in unknown sample was estimated using glutathione standard curve [15]. The total flavonoids content of the sample was determined by a Pharmacopoeia method using Rutin as a reference compound. The blank was prepared by diluting 100µl of plant extract in one drop of acetic acid and 2.5ml with ethanol and expressed as rutin equivalents [16]. The tannin content of the sample was determined using tannic acid as a standard [17]. Total alkaloids were estimated in methanol extract and the complex formed with bromocresol green in the chloroform layer was measured at 470 nm using atropine as standard [18]. Cardiac glycosides were extracted in 70% alcohol from the powdered sample, filtered followed by the addition of Buljet's reagent and the percentage of cardiac glycoside was calculated [19, 20]. Total saponins was determined by gravimetric method by the method described by Harbone JB, 1973 [21]. Ascorbic acid was extracted on fresh basis using 6% HPO_3 solution in both the MI varieties using the method described by Ranganna S, 1999 [22]. Terpenoids were determined spectrophoto-metrically using linalool as standard [23]. Rhein served as a standard for the determination of anthraquinone glycosides [24].

Antioxidant Activity:

Radical Scavenging Activity: The method followed is prescribed by Gulluce *et al.,* (2006) [25]. Briefly, about 1ml of 1mM DPPH in methanol (containing 100-500µg of dried extract) was taken. The mixture was than vortexed vigorously and left for 30min at room temperature in dark. The absorbance was measured at 517nm. The DPPH radical scavenging activity % was calculated according to the following equation.

Radical scavenging activity (%) = Absorbance of control-absorbance of sample ×100 / Absorbance of control

Anti-cancer Activity:

***In-vitro* Cytotoxic Activity by MTT Assay:** The anti-cancer activity was performed in the MI variety which exhibited better phytochemical profile. The *in-vitro* cytotoxicity of MI was carried out in HT-29 colon cancer cell line by the MTT assay. The viability of the cells was checked under an inverted microscope to obtain a cell count of 8×10^6 cells and centrifuged. Further, 5×10^4 cells of HT-29 were seeded in each well of a 96 well plate and incubated for 24 h at 37 °C and 5% CO_2. The test samples (methanol and aqueous extracts of MV1) were added at concentrations ranging

between 0-320 µg/ml in DMEM cell culture medium and incubated for 24 h. After incubation with test samples, 100 µl of 5 mg/ml MTT in 1X PBS was added to each well and incubated for 4 hours under dark condition. The MTT reagent was discarded by pipetting without disturbing cells and 100µl of DMSO (100%) was added and incubated for 15 min to rapidly solubilize the formazan crystals. The absorbance was measured at 590nm in a multi-plate reader (Tecan Spectra Fluor Plus, MTX Lab Systems). The cytotoxicity of the extracts was calculated using the formula

% Inhibition = 100 – (OD of sample / OD of Control) × 100

Detection of Apoptosis by Annexin V-FITC Staining Method: The extract that exhibited higher cytotoxic effect in the selected MI variety was further screened for apoptotic activity. The HT-29 cell lines were seeded at a density of 1×10^6 HT-29 cells per well in a 6-well plate using DMEM cell culture medium and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h to form a cell monolayer. After 24 h, the medium was aspirated and treated with 160 µg/mL concentration of the extract and compared with controls *viz*., control without treatment and positive control Colchicine (20µM). The cells were treated for 24 h. After treatment, the cells were washed, trypsinized, and centrifuged for 8 min at 1800rpm. The mixture was centrifuged and the supernatant was discarded. The cells were washed twice with cold PBS and re-suspended in Binding Buffer (1X) at a concentration of ~1×10^6 cells/mL.100µL of the solution (~1×10^5 cells) was transferred to a 5mL FACS tube. 5µL Annexin V-FITC was added and incubated at 4 °C for 30 min. Further, 5µL propidium iodide (PI) were added to the tubes. The cells were mixed gently and incubated for 5 min at RT in the dark and cells were analyzed by flow cytometer.

Statistical Analysis: The statistical analysis was conducted using the SPSS version 16 statistical tool. Independent sample t-test was applied to compare means of quantitative analysis. One-way Analysis of variance (ANOVA) with Tukey's post-hoc test was applied to compare means of the quantitative analysis of extracts, DPPH activity and MTT assay. The coefficient of determination (R^2) between the concentration of extracts and DPPH activity was analyzed. Statistical significance was accepted at $p\leq0.05$. The IC_{50} values were calculated from log dose-response curves using GraphPad Prism software (version 6).

RESULTS: The total yield of leaf powder of MV1 and MS36 obtained after drying were 55.12% and 71.33%, respectively. The aqueous (15.6%) and ethanol (13.2%) extract of MS36 had better yield than MV1 (14.40% and 13%) whereas the yield of methanol (14.6%) extract was higher in MV1 than MS36 (14.2%). The total yield of all the extracts ranged between 13-16%. The results indicate that the yield of extract increases with increasing polarity of solvent. The maximum yield was obtained in aqueous extract (MS36) followed by methanol (MV1) and ethanol respectively.

Qualitative Screening of Phytochemicals in *Morus indica* Varieties: The MI varieties *viz*., MV1 and MVS36were screened for phyto-chemicals such as saponins, terpenoids, alkaloids, tannins, flavonoids, phenols, phytosterols and glycosides using both extracts and dried sample **Table 1**.

TABLE 1: QUALITATIVE SCREENING OF PHYTOCHEMICALS IN *MORUS INDICA* VARIETIES

	Dry basis		Methanol extract		Ethanol extract		Aqueous extract	
Phytochemicals	**V1**	**S36**	**V1**	**S36**	**V1**	**S36**	**V1**	**S36**
Saponins	+ve	+ve	-	-	-	-	-	-
Terpenoids	+ve	+ve	-	-	-	-	-	-
Alkaloids	-ve	-ve	-	-	-	-	-	-
Tannins	-	-	+ve	+ve	+ve	+ve	+ve	+ve
Flavonoids	-	-	+ve	+ve	+ve	+ve	+ve	+ve
Polyphenols	-	-	+ve	+ve	+ve	+ve	+ve	+ve
Phytosterols	-	-	+ve	+ve	+ve	+ve	+ve	+ve
Glycosides	-	-	+ve	+ve	+ve	+ve	+ve	+ve

"+ve" – presence of phytochemical, "-ve" – absence of phytochemical, "-" Not analyzed

The qualitative analysis showed the presence of phytochemicals such as saponins, flavonoids, tannins, terpenoids, phenols, phytosterols and glycosides in both the varieties except for alkaloids.

Quantitative Analysis of Phytochemicals in *Morus indica* Varieties: The quantitative phytochemical analysis of MI varieties *viz*., MV1 and MVS36 included estimation of polyphenols, glutathione, tannins, cardiac glycosides, saponins, anthraquinone glycosides, ascorbic acid, flavonoids, alkaloids and terpenoids. The quantitative screening of phytochemicals was conducted in extracts or dried sample as per the protocol. The quantitative analysis of phytochemicals in MV1 and MS36 on dry basis is given in **Table 2** and of the extracts in **Table 3**.

TABLE 2: QUANTITATIVE PHYTOCHEMICAL PROFILE OF *MORUS INDICA* VARIETIES

Phytochemicals	MV1 (g/100g)	MS36 (g/100g)	t-test	p-value
Saponins	2.66±0.02	2.13±0.121	7.470	0.015
Tannins	10.40±0.18	10.70±0.06	-2.727	0.053
Polyphenols	0.546±0.02	0.441±0.01	5.892	0.004
Cardiac glycosides	0.08±0.002	0.07±0.001	11.885	0.0001
Glutathione	0.257±0.002	0.192±0.005	19.902	0.0001
Anthraquinone glycosides	0.464±0.01	0.434±0.009	3.840	0.018
Ascorbic acid*	0.013±0.001	0.016±0.000	-2.828	0.047

The values are expressed as g/100g of sample on dry basis. Note: * ascorbic acid was estimated on fresh basis. Independent sample t-test was applied to compare means and the values are significant at the level $p \leq 0.05$.

TABLE 3: QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS IN *MORUS INDICA* EXTRACTS

Extracts	Flavonoids (mg/g)	Alkaloids (mg/g)	Terpenoids (mg/g)
MV1A	28.326 ± 0.097^{e}	0.066 ± 0.011^{b}	0.416 ± 0.050^{ab}
MS36A	25.950 ± 0.290^{c}	0.051 ± 0.002^{a}	0.350 ± 0.079^{ab}
MV1ME	29.413 ± 0.100^{f}	0.063 ± 0.001^{ab}	0.343 ± 0.100^{ab}
MS36ME	27.133 ± 0.116^{d}	0.071 ± 0.001^{b}	0.250 ± 0.120^{a}
MV1ET	20.20 ± 0.389^{b}	0.070 ± 0.0^{b}	0.546 ± 0.025^{b}
MS36ET	18.143 ± 0.116^{a}	0.061 ± 0.002^{ab}	0.463 ± 0.049^{ab}

One-way ANOVA with Tukey'spost-hoc test was applied to compare mean values of the extracts. Values a, b, c, d, e, f differ significantly ($p<0.05$). A – aqueous extract, ME – methanol extract, ET – ethanol extract.

From the results of the quantitative analysis of phytochemicals, it can be observed that the tannins were in the highest amount (10.40 ± 0.18g in MV1 and 10.70 ± 0.06g in MS36) in both the varieties followed by saponins (2.66 ± 0.02g in MV1 and 2.13 ± 0.121g in MS36) and polyphenols (0.546 ± 0.02g in MV1 and 0.441 ± 0.01g in MS36). The glutathione, anthraquinone glycosides, cardiac glycosides and ascorbic acid were present in lesser amounts. Antioxidants such as glutathione and ascorbic acid content of MV1 were 0.257 and 0.013 mg/100g; and of MS36 were 0.192 and 0.016 mg/100g respectively. The glutathione content of both the hybrid varieties were higher than the reported values of Roy L.G *et al.*, (2010) [26] in dehydrated mulberry leaves (*Morus indica*) *i.e.*, 152mmol or 0.152mg/100g of glutathione.

The alkaloid, flavonoid and terpenoid contents were estimated in the extracts and the results are depicted in **Table 3**. The alkaloid contents of the aqueous (A), methanol (ME) and ethanol extracts (ET) ranged between 0.06-0.07mg/g. The results of qualitative analysis on dry basis showed absence of alkaloids which can be extrapolated to quantitative analysis which showed negligible amounts of alkaloids in the extracts. The terpenoid content of the MI extracts ranged between 0.25-0.546mg/g. The ethanol extract had the highest terpenoid content in both the varieties compared to other extracts. The total flavonoid content estimated in MV1 and MS36 ranged between 18.143 to 29.413mg/g extract, respectively which was higher than other phytochemicals estimated in the extracts.

The results were comparable to the values reported by Iqbal S *et al* (2012) [27] in *Morus alba* and *Morus rubra* which were 26.41 mg/g to 31.28 mg/g of flavonoids respectively on dry basis.

Among the extracts, MV1ME (29.92 mg/g), MV1ET and MS36ME (0.07 mg/g); and MV1ET (0.52 mg/g) had the highest flavonoid, alkaloid and terpenoid contents respectively. Overall, MV1 had a significantly ($p<0.05$) better phytochemical profile than MS36.

Radical Scavenging activity of *Morus indica* Varieties: The radical scavenging activity (RSA) by DPPH method gives an over view on the free

radical scavenging power of the medicinal plant by neutralizing the odd electron of DPPH. The radical scavenging activity is expressed as %. The RSA of MI varieties is given in **Table 4.**

TABLE 4: RADICAL SCAVENGING ACTIVITY OF *MORUS INDICA* VARITIES BY DPPH METHOD

	%DPPH activity in the extracts (R^2-value)					
Concentration (µg/ml)	**MV1A (0.9559)**	**MS36A (0.5117)**	**MV1ME (0.9281)**	**MS36ME (0.8807)**	**MV1ET (0.9581)**	**MS36ET (0.9865)**
100	14.82±2.19^{a}	18.28±8.59^{ab}	26.59±9.88^{b}	12.11±3.21^{a}	13.44±1.94^{a}	7.29±1.53^{a}
200	23.75±4.18^{a}	20.81±1.43^{a}	40.00±3.98^{b}	38.33±4.80^{b}	27.10±6.40^{a}	19.86±0.26^{a}
300	26.86±4.30^{b}	14.92±2.18^{a}	57.08±0.63^{d}	63.12±1.73^{e}	27.00±2.92^{b}	39.19±1.30^{c}
400	34.74±8.43^{b}	20.42±6.17^{a}	66.95±1.64^{d}	64.0±1.47^{d}	46.51±5.59^{bc}	47.64±7.72^{c}
500	40.83±6.08^{b}	22.09±1.45^{a}	68.20±0.84^{d}	64.12±2.11^{d}	55.19±4.41^{c}	62.63±2.09^{cd}

MV1A – Aqueous extract of V1, MS36A – Aqueous extract of MS36, MV1ME – Methanol extract of V1, MS36ME - Methanol extract of S36, MV1ET - Ethanol extract of V1, MS36ET - Ethanol extract of S36. Superscripts a, b, c, d, e…differ significantly ($p<0.05$) between the extracts at different concentrations.

Among the extracts, MVIME (68.20%, R^2=0.9281) had the highest activity followed by MS36ME (64.21%, R^2=0.8807). The lowest activity was observed in MS36A (22.09%, R^2=0.5117). The RSA followed the order MV1ME > MS36ME > MS36ET > MV1ET > MV1A > MS36A. The %DPPH activity of the extracts had good correlation with the concentration of extracts. The results of the % DPPH activity of MI in our study were similar to the antioxidant potential of *Morus nigra* L. reported by Souza GR *et al.*, (2017) [28] where ethanol and ethyl acetate extracts exhibited higher activity than other solvent extracts.

Since MV1 had a better phytochemical profile, especially with compounds known for exhibiting anti-cancer effect, it was chosen for further analysis. Since, the study aimed at utilizing MI as a potential nutraceutical source, ethanol extract which generally shows lower extractable solids was eliminated from screening the anti-cancer effect.

Anti-cancer Potential of *Morus indica* Varieties: After screening the antioxidant potential of MI hybrid varieties, the aqueous (MV1A) and methanol (MV1ME) extracts of MV1 were studied for the cytotoxic ability in HT-29 colon cancer cell line.

***In-vitro* Cytotoxic Effect of *Morus indica* V1 in HT-29 Cell Line:** The cytotoxic effect of MV1A and MV1ME was evaluated on HT-29 cells using MTT assay **Table 5**. The cell lines were exposed to doses ranging between 10– 320 µg/mL and the IC_{50}values were calculated after the 24 h treatment. The extracts exhibited significant dose-dependent inhibition of growth on HT-29 cells with IC_{50} values of 169.7 µg/ml (MV1ME) and 55.01 µg/ml (MV1A) respectively. Some S *et al.*, (2019) [29], reported maximum cytotoxicity of 41% and 49% by MV1 aqueous extract based silver nanoparticles at 250µg/ml concentration on HepG2 and WRL-68 human cancer cell lines respectively with a IC_{50} higher than 250 µg/ml. In our study, the aqueous extract of MV1 showed high cytotoxic effect on colon cancer cell line with very low IC_{50} value. Since MV1A exhibited the least IC_{50} value, it was chosen for screening apoptosis inducing potential.

TABLE 5: *IN-VITRO* CYTOTOXICITY STUDY OF EXTRACTS OF MV1 IN HT-29 COLON CANCER CELL LINE BY MTT ASSAY

Extracts	Conc. µg/ml	% Inhibition	IC_{50}
MV1ME	10	8.740±0.01	169.7
	20	16.523±0.02	
	40	21.646±0.05	
	80	27.633±0.03	
	160	47.203±0.20	
	320	56.663±0.005	
MV1A	10	5.156±0.05	55.01
	20	12.390±0.04	
	40	29.220±0.09	
	80	47.700±0.05	
	160	61.556±0.11	
	320	70.846±0.03	
Positive control	10	40.776±0.01	NA
	20	44.460±0.04	

One-way ANOVA with Tukey's post-hoc test was applied to compare mean values of the groups. All the values differ significantly from each other at level $p<0.05$. NA = Not applicable; MV1A- Aqueous extract of V1, MV1ME – Methanol extract of V1.

Apoptotic Effect of *Morus indica* V1 Aqueous Extract in HT-29 Cell Line: The Annexin/PI assay was carried out to confirm the ability of the extract to induce early and late apoptosis. Unlike necrosis, apoptosis is an important cell death mechanism that does not trigger an inflammatory

response and is a protective mechanism that maintains tissue homeostasis by removing ailing cells. Cancer cells exhibit resistance to apoptosis in order to sustain their uncontrolled proliferation. The plant extract should possess the ability to induce apoptosis and cease proliferation. The process of apoptosis induced by the test samples were detected using Annexin V-FITC/ PI staining of the cells by flow cytometric method. The percentage of live and dead cells in untreated, positive control and experimental group is given in **Fig. 1.**

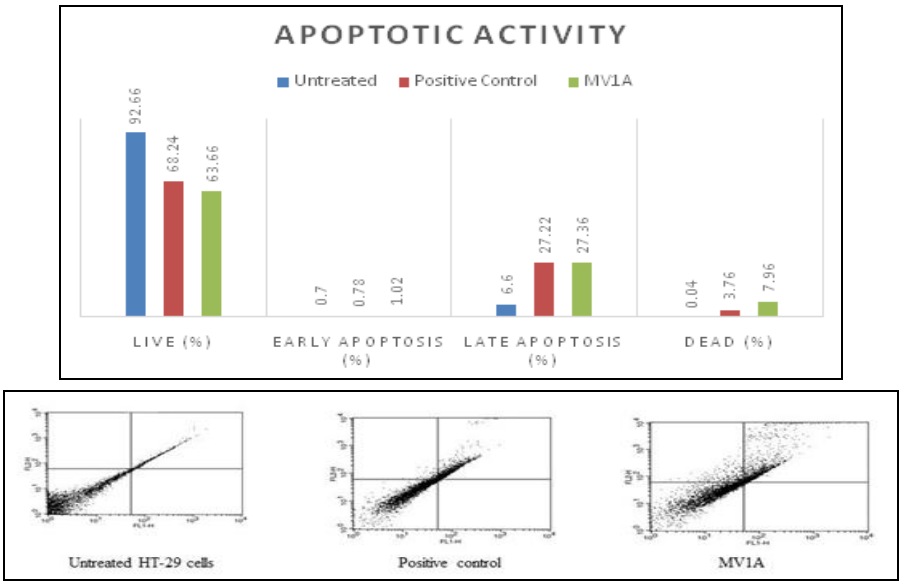

FIG. 1: DETECTION OF APOPTOTIC ACTIVITY OF AQUEOUS EXTRACT OF MV1 IN HT-29 CELL LINE.
Note: Positive Control: HT-29 cells treated with 20µM Colchicine; MV1A: HT-29 cells treated with 160µg/mL of aqueous MV1 extract

The apoptosis-inducing potential of the MV1A extract was tested by Annexin V-FITC/ PI Staining of HT-29 cells and evaluated using flow cytometry. The values obtained were compared with the control (untreated cells) and positive control (colchicine 20µM/mL). The cells were treated with 160 µg/mL of MV1A for 24h. **Fig. 1** depicts the results after 24 h of incubation, wherein HT-29 cells had majorly undergone late apoptosis after treatment with positive control and MI extract. Live cells were found to be higher in untreated cells (92.66%) whereas 68.24% in colchicine and 63.66% in MV1A extract. The percentage of early apoptotic cells were higher in MV1A treated cells (1.02%) followed by colchicine (0.78%) and untreated cells (0.70%). The percentage of late apoptotic cells were found to be higher in MV1A (27.36%) compared to untreated (6.60%) and, also MV1A had exhibited similar activity as that of positive control (27.22%). The percentage of dead cells in untreated, positive control and MV1A were 0.04%, 3.76% and 7.96%, respectively. The extract of MV1A had the potential to induce apoptosis in HT-29 cells at a dosage of 160 µg/mL and its activity was comparable to commercial drug-colchicine.

DISCUSSION: The results of the phytochemical screening were comparable with studies reported in the literature [7], however, the presence of alkaloids in the hybrid varieties of MI were low. Many bioactive compounds have shown specific role as anti-cancer agents. The presence of polyphenolic compounds [30, 31], tannins [32], anthraquinone glycosides [33], cardiac glycosides [34] and terpenoids [35] which specifically are known to possess anti-cancer properties makes MI hybrid varieties a novel source of nutraceutical compounds. Though certain compounds are present in smaller amounts, they work synergistically and exhibit their biological role. Both the varieties of *Morus indica* had higher total phenolics than *Morus alba* as reported by Sánchez-Salcedo, E. M *et al.*, (2015) [36]. MV1 had higher terpenoid content than MS36 among the extracts. The anti-cancer properties of terpenoids have been demonstrated in various medicinal plants and has exhibited a protective role majorly in breast and gastrointestinal cancers [37- 41]. Selection of solvent for extraction of bioactive compounds of target plays a major role in determining the biological activity and therefore, studying the nature of target compounds becomes vital.

The selective cytotoxic effects of other species of Morus in cancer cell lines of colon [42], lung [43] and cervix [44] have been reported. However, aqueous extract of MV1 in our study has exhibited the lowest IC_{50} when compared to values reported in literature.

The result of apoptosis of MV1A on HT-29 cells was contradicting to the results reported by Naowaratwattana, W *et al* (2010) [45], where aqueous extract of *Morus alba* leaves extract exhibited poor apoptotic activity than the methanol extract. This observation could possibly be due to changes in the phytochemical composition between species and the geographical location in which these plants are grown. The apoptotic activity of other species of Morus has been reported in literature [9, 41, 46].

Although, other species of Morus have been well explored for its anti-cancer property, there are very few studies which report the antioxidant and anti-cancer potential of *Morus indica*. To the best of our knowledge, this study is one among very few studies reporting the anti-cancer activity of hybrid variety of *Morus indica.*

CONCLUSION: The aqueous extract of MV1 variety has high cytotoxic effect and can therefore be a safe and novel source of nutraceutical with anti-cancer property. The study paves a way to further evaluate the anti-cancer potential of MV1 variety in *in-vivo* model systems.

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