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In-vitro Cytotoxic Activity of Fractions Isolated from *Vigna Mungo* and *Vigna Radiata*

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ABSTRACT

Pulses and legumes have been gaining interest because they are excellent source of bioactive compounds. Aim of the present investigation is to evaluate in-vitro cytotoxic activity of the fractions isolated from Vigna mungo and Vigna radiata on MCF-7 human breast cancer cells. Seeds of both the plants were selected and fractions were isolated by using column chromatography technique.Preliminary identification of the isolated fractions was done by preliminary tests. Isolated fractions were subjected to extracted ion chromatogram quantification. In-vitro cytotoxic activity of four fractions were evaluated by using MTT assay and SRB assay. Two fractions from each of Vigna mungo and Vigna radiata were isolated. Vigna mungo fractions shows presence of rutin and quercetin. Rutin was present in higher concentration in fraction 1. Fraction 3 and 4 of Vigna radiata showing presence of gallic acid, cinnamic acid, syringic acid, Kaempferol and quercetin. Fractions of both plants showing dose dependent cytotoxic activity on MCF-7 cells in MTT assay.IC 50 values of F1 and F2 are 19.053 ± 1.003 and 21.5224 ± 4.871 respectively. IC 50 values of F3 and F4 are 77.553± 0.778 and 13.7639 ± 0.7853 respectively. Fraction 3 and fraction 4 of Vigna radiata and Vigna mungo also displayed significant action against MCF-7 human breast cancer cells at 40 and 80 µg/ml concentration. Isolated fractions of both plants showed dose dependent cytotoxic activity against cell line by using SRB assay. Cytotoxic effect of fractions of Vigna mungo and Vigna radiata is possibly due to presence of flavonoids and phenolics in isolated fractions as reported literature suggest that flavonoids and phenolic compounds are responsible for potential anticancer activity.

Key words: *Vigna mungo, Vigna radiata*, MTT assay, SRB assay, cytotoxicity

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INTRODUCTION

The search for natural bioactive compounds (NBCs) with potential for the treatment and prevention of human diseases and to meet other needs is currently a key topic in many laboratories and industries. These compounds efficiently interact with proteins, DNA, and other biological molecules to produce a desired outcome, which could be exploited for designing natural products-derived therapeutic agents. Pharmaceutical and food domains share a similar interest to obtain and characterize new NBCs which can be used as drugs, functional food ingredients, or nutraceuticals. It is important to notice that more than 2/3 of the world population still relies on medicinal plants for their primary pharmaceutical care. In the last 25 y about 60% to 70% of newly approved drugs on cancer and infectious diseases were derived from NBCs.

In the traditional system of medicine this Vigna genus is mainly used in the treatment of liver disorders, ulcers, to decrease the weight, and also used in hormonal balance. [1]This genus consists of several species like *Vigna mungo* (Urd bean, Black gram, black lentil),*Vigna radiata* (Mung Bean, Green Gram, Golden Gram, Mash Bean, Green Soy),*Vigna unguiculata* (Cowpea, Crowder Pea, Southern Pea), *Vigna*

*angularis (*Adzuki Bean, "red bean") and other relative species. [2]Grain legumes are being cultivated in India since time immemorial. Legumes are considered as a "poor man's meat".Green gram (*Vigna radiata*) and black gram(*Vigna mungo)* are two of the most important food legumes grown and consumed in India. Legumes contain greater varieties of toxic constituents than any other plant family. The toxic compounds consist of some flavonoids, alkaloids, tannins, cyanogenic compounds, phytate and trypsin inhibitors.[3] Aureol, coumestrol, cyclokievitone, dalbergioidin, 2,3-dehydrokievitone,genistein, 2'-hydroxygenistein, isovitexin, kievitone, myrtillin, phaseol, phaseollidin, vitexin etc were reported flavonoids from seeds of *Vigna radiata*. Others constituents from the seed includes ß-sitosterol, stigmasterol, soyasapogenol C, 1,4-butanediamine, 3-(carboxymethylamino) propanoic acid, 1H-imidazole,spermidine, spermine, amino acids and peptides[2]Kite *et al* has worked on the use of hyphenated techniques in comparative phytochemical studies of legumes. The use of GC-MS has provided an extensive data set on the occurrence of quinolizidine alkaloids in legumes GC-MS also provides the means to separate the numerous isomeric forms of polyhydroxyalkaloids and hydroxypipecolic acids as their volatile trimethylsilyl derivatives. LC-MS is enabling the metabolic profiles of intact flavonoid glycosides to be obtained from small fragments of material while recent methods to analyse non-protein amino acids by LC-MS without derivatisation hold much promise in surveys of these important taxonomic characters.[4]Black gram flour contained phenolic acids like Gallic, protocatechic, gentisic, vanillic, syringic, caffeic and ferulic acids. As black gram and its fractions are rich in antioxidant compounds and nutrients, they may have potential applications as nutraceuticals and functional food ingredients in various processed foods for the improvement of health benefits.[5]Mung bean consists of antinutrients like phytate and polyphenols [6]Milled fractions of black gram shows distribution of *C*-glycosyl flavones and higher concentrations of *C*-glycosyl flavones are present in husk fractions. These compounds were identified as vitexin and isovitexin. *C*-glycosyl flavones from black gram husk exhibited anticancer activity and protected DNA and erythrocytes from oxidative damage.[7]Neutral detergent fiber was isolated from the black gram. NDF contains hemicellulose, cellulose, lignin, cutin and silica. Hemicellulose is present in higher concentration and it is considered as most active constituent. Isolated NDF showed hypolipidemic, hypoglycemic and anticolon cancer activity. [8]Polyphenols and mung bean trypsin inhibitor fragment showed antitumor activity on tongue squamous cell carcinoma cell line CAL27 and several other cancer cell lines [9].

Cancer is the second leading cause of death globally and is estimated to account for 9.6 million death in 2018. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervix and thyroid cancer are the most common among women.Cancers of oral cavity and lungs account for over 25% of cancer deaths in males and cancer of breast and oral cavity account for 25% cancers in females[10]

Reported literature suggested that both of these plants consist of phytochemicals that are active against tumor cells. Aim of the present study is evaluation of cytotoxic activity of fractions isolated from*Vigna mungo* and *vigna radiata* by using invitro MTT and SRB assay method on MCF-7 human breast cancer cell lines.

MATERIAL AND METHODS

Collection and Authentication of Plant Material:

Plant material was collected from local farmers located near Alandi area Pune. Plant specimens were prepared and sent to the Botanical Survey of India, Pune for authentication.

Extraction of Plant Material:

Collected plant material was washed properly and foreign organic matter was separated. Seeds of *Vigna mungo*and *Vigna radiata* were coarsely powdered for extraction. Coarse material was loaded into Soxhlet apparatus and defatted using Petroleum ether solvent. Exhausted powder was air dried and again loaded into Soxhlet apparatus. Ethanol water was used as a solvent for extraction. Hydro-alcoholic extracts were collected and concentrated by using Rotary Vacuum evaporator. Dried extract was kept in desiccator for storage.

Separation of fractions

Extract obtained from both the plants were subjected to column chromatography. Glass column was taken and stationary phase was prepared by pouring slurry of Silica Gel 60-120 in n-hexane solvent. Air bubbles were removed by tapping on column. Before pouring slurry cotton wool and 1cm thick Silica was added. After silica loading sample was added in column. Sample was prepared by mixing it with silica gel and then trituration. First n-Hexane wash was given and then polarity was decreased by using Ethyl acetate by ratio like 95:5, 90:10, 85:15, and 80:20 up to 10:90.

Phytochemical analysis of isolated fractions

Chemical constituents present in isolated fractions were identified by preliminary phytochemical tests. Test for Glycosides, saponins, alkaloids, phenolic, flavonoids etc. were performed. Isolated fraction were subjected to extracted ion chromatogram based quantification of the some phenolic compounds.

In vitro cytotoxicity study:

MTT assay

100 µl of cell suspension of density 1×10^4 cells/well was placed into each well of 96-well plates and incubated for 24 h. Fractions of *Vigna mungo* and *Vigna radiata* were dissolved in DMSO and added (10-80 µg/mL) to cultured cells in 96 well plates and incubated for 24 h. Then, the medium was removed and washed with 200 µl phosphate buffered saline (PBS). Added 100 µL of the tetrazolium dye (MTT) solution (1 mg/mL in PBS) to each well of 96 well plates and incubated for 4 h at 37°C. MTT reagent was discarded by inverting the microplates. Formed formazan crystals were dissolved by adding 100 µL of DMSO. The plate was placed in a plate shaker for 5 min. then optical density was read on a microplate reader at 540 nm. The experiment was performed in triplicates. [11]

Sulforhodamine B assay

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5 % CO2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs.Experimental drugs were initially solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquote of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml. After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (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 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mMtrizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100.[12,13]

Statistical Analysis

All the experiments were performed in triplicates. Data were represented as mean ± SEM values. Statistical analysis of the data were performed by one-way ANOVA (Graph Pad Prism 8.1.1, Graph Pad Software, Inc., California) followed by Tukey's test.

RESULTS

Plant specimens were identified as follows with voucher specimen numbers

TABLE 1 AUTHENTICATION OF PLANT SPECIES

Specimen No.	Plant Name	Family
DJS 04	*Vigna radiata* L. R. Wilczek	Fabaceae
DJS 05	*Vigna Mungo* L. Hepper	Fabaceae

Two fractions from seeds of *Vigna mungo*(F1 and F2) and two from *Vigna radiata.*(F3 and F4)were isolated*.* Preliminary phytochemical tests suggest presence offollowing constituents

TABLE 2 PRELIMINARY PHYTOCHEMICAL ANALYIS

Fraction	Description	Constituents
F1	Light green, semisolid	Glycosides, Phenolic, Saponins
F2	Yellowish green, semisolid	Glycosides, Phenolic, Saponins
F3	Yellowish green, semisolid	Glycosides, Phenolic, Saponins
F4	Dark green	Glycosides, Phenolic, Saponins

Extracted ion chromatogram:

Isolated fractions were subjected to extracted ion chromatogram-based quantification of some phenolic compounds. Extracted ion chromatogram of VM fraction 1 shows presence of rutin and quercetin in the

concentration of 84.04 µg/10 mg and 0.299 µg/10 mg of extract. VM fraction 2 shows rutin (0.072 µg/10 mg) quercetin (0.017 µg/10 mg). Gallic acid, syringic acid, quercetin and kaempferol are present in VR F3. Syringic acid was found to be present in higher concentration. Fraction 4 of Vigna radiata shows presence of syringic acid, cinnamic acid and quercetin.

TABLE 3 EXTRACTED ION BASED QUANTIFICATION

Sample	Gallic acid	Syringic acid	Rutin	Cinnamic acid	Quercetin	Kaempferol
VM F1	--	--	84.0449825	--	0.29962985	--
VM F2	--	--	0.07151655	--	0.01667754	--
VR F3	0.03788599	2.62782082	--	--	0.02381961	1.15413231
VR F4	--	0.70004337	--	0.01696732	0.01657982	--

Results are given in µg/10 mg of the fractions.

MTT assay:

In this study *Vigna mungo* fraction I and fraction II significantly reduced cell viability of MCF-7 breast cancer cells in 40 and 80 µg/ml concentration when compared with the control group. F1 40 treated group reduced cell viability up to 77.61% and F1 80 treated group reduced it to 77.24%. F2 80 showed 44.01% cell viability. Fraction II shown more cell inhibition than fraction I. This showed that fraction II has more significant cytotoxic activity than fraction I. IC 50 values of F1 and F2 are 19.053 ± 1.003 and 21.5224 ± 4.871 respectively.

TABLE 4 RESULTS OF MTT ASSAY *VIGNA MUNGO* VM FRACTION 1 AND FRACTION 2

Group	Treatment	VM Fraction 1		VM Fraction 2	
		Absorbance	**% Cell viability**	**Absorbance**	**% Cell viability**
I	Control	0.335±0.001	100	0.259 ±0.019	100
II	F 10	0.326±0.005	97.31	0.230 ±0.005	88.80
III	F 20	0.293±0.013*	87.46	0.180 ±0.006*	69.49
IV	F 40	0.260±0.006***	77.61	0.147 ± 0.029**	56.75
V	F 80	0.258±0.001***	77.24	0.114 ± 0.002***	44.01

All values are expressed as mean ± SEM. n = 3. All data were subjected to One Way ANOVA followed by Tukey's multiple comparison test. F 10 = 10µg/ml, F 20 = 20µg/ml, F 40 = 40µg/ml, F 80 = 80µg/ml of VM fraction 1 and 2 treated groups. Control group treated with no fraction or drug. Group II, III, IV, V are compared with group I. *p<0.05, **p<0.01, ***p<0.001

At 540 nm VR fraction 3 showing significant absorbance in all four groups. Group II and III showing **p<0.01 significance while group IV and V showing higher significance p<0.001 after comparison with control group. After 48 hours % cell viability was decreased in dose dependent manner in cells treated with fraction 3 and 4 of *Vigna radiata.* VR F3 shown 60.61% cell viability when treated with VR F3 80 while VR F4 decreased viable cells up to 50.19%. IC 50 values of F3 and F4 are 77.553± 0.778 and 13.7639 ± 0.7853 respectively. Both the fractions are showing significant cytotoxic activities.

TABLE 5 RESULTS OF MTT ASSAY *VIGNA RADIATA* VR FRACTION 3 AND FRACTION 4

Group	Treatment	VR Fraction 3		VR Fraction 3	
		Absorbance	**% Cell viability**	**Absorbance**	**% Cell viability**
I	Control	0.259 ±0.019	100	0.259 ± 0.019	100
II	F 10	0.186 ± 0.004 **	71.81	0.241 ± 0.003	93.05
III	F 20	0.185 ± 0.004 **	71.42	0.147 ± 0.020 ***	56.75
IV	F 40	0.172 ± 0.003 ***	66.40	0.137 ± 0.003 ***	52.89
V	F 80	0.157 ± 0.005 ***	60.61	0.130 ± 0.003 ***	50.19

All values are expressed as mean ± SEM. n = 3. All data were subjected to One Way ANOVA followed by Tukey's multiple comparison test. F 10 = 10µg/ml, F 20 = 20µg/ml, F 40 = 40µg/ml, F 80 = 80µg/ml of VR fraction 3 and 4 treated groups. Control group treated with no fraction or drug. Group II, III, IV, V are compared with group I. *p<0.05, **p<0.01, ***p<0.001

SRB Assay

In SRB assay percentage of cell growth was determined after treatment of extracted fraction relative to the control group. Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Fraction I and fraction II of *Vigna mungo* displayed substantial action against MCF-7 human breast cancer cells at 40 and 80 µg/ml concentration. Fraction 3 and fraction 4 of *Vigna radiata* also displayed significant action against MCF-7 human breast cancer cells at 40 and 80 µg/ml concentration. Isolated fractions of both plants showed dose dependent cytotoxic

activity against cell line by using SRB assay.

TABLE 6 SRB ASSAY % CELL VIABILITY

Group	% Cell viability			
	VM F1	VM F2	VR F3	VR F4
Control	100 ± 1.00	100.00 ± 1.000	100.00 ± 1.000	100.00 ± 1.000
10 µg/ml	98.40 ± 0.781	99.47 ± 0.929	99.13 ± 1.106	95.07 ± 5.173
20 µg/ml	98.03 ± 1.250	97.50 ± 1.100	94.10 ± 3.904	88.00 ± 1.000*
40 µg/ml	96.40 ± 1.929*	89.33 ± 3.055**	93.53 ± 4.007	82.30 ± 4.359**
80 µg/ml	94.20 ± 1.153**	86.33 ± 3.512***	86.17 ± 3.502**	77.17 ± 6.397***

Effect of various concentrations of different fractions of plant material on MCF-7 breast cancer cells. Results were stated as Mean ± SD of tests performed in triplicates. The values were expressed as * P < 0.05, ** P < 0.01 and *** P<0.001. Values significance was calculated by comparison of % cell viability of fraction group with control group by using one-way ANOVA followed by Tukey's test.

DISCUSSION

The general purpose of the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay is to measure viable cells in relatively high throughput (96-well plates) without the need for elaborate cell counting. Therefore the most common use is to determine cytotoxicity of several drugs at different concentrations. The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals, which can be solubilized for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 540nm. For drug sensitivity measurements the OD values ofwells with cells incubated with drugs are compared to the OD of wells with cells not exposed to drugs[14]Variable results may obtain in MTT assay as the formation of colorrelies on the activity of the mitochondria and if the function of these is inhibited by differences in cellular factors like NADH, glucose and other factors variation may occur. [15]SRB assay is based on uptake of the negatively charged pink amino xanthine dye, sulphorhodamine B (SRB) by basic amino acids in the cells. The greater the number of cells, the greater amount of dye is taken up and, after fixing, when the cells are lysed, the released dye will give a more intensecolour and greater absorbance [16].

Black seed-coats and the purple-red hypocotyls of Vigna plants found to contain different anthocyanin svizdelphidin 3-glucoside, delphinidin 3-p-coumaroyl glucoside and cyanidin 3- glucoside. Leucocyanidin and leucodelphinidin are present in the seed coats of plant. Two glycoflavones vitexin and isovitexin were detected in all of the seed-coats. Three flavonol glycosides Robinin, Kaempferol 3-rutinoside and Kaempferol 7-rhamnoside were found in the leaves of black gram. [17]Proanthocyanidin, delphinidin and cyanidin are present in the seeds. Kaempferol appears to be the most prevalent flavonoid in the *Vigna mungo.* [18]Adesanya et al identified 16 isoflavonoids from *Phaseolus mungo* (*Vigna mungo*) by their UV, MS and PMR characteristics. Five compounds from these genistein, 2'-hydroxygenistein, kievitone, dalbergioidin and demethylvestitol are reported to be present in Leguminosae as a phytoalexins. Five compounds are known to occurring only in the genus Phaseolus. These were identified as the isoflavone-2'-hydroxydaidzein, the isoflavanonescyclokievitone, 5-deoxykievitone, 2'-hydroxydihydrodaidzein and the coumestanaureol. Isoflavanone isoferreirin and the pterocarpanglycinol were two *P. mungo* isoflavonoids. Three novel natural isoflavanones which were characterized as 4'-0-methylkievitone, cyclokievitone hydrate and 5-deoxykievitone hydrate along with reported kievitone. [19]Hypocotyl or epicotyl tissue of the black gram seeds shows presence of phytoalexins which are dalbergioidin, kievitone, phaseollidin and one unidentified substance. [20]Sharma *et al* isolated isoflavones from black gram. Black gram shows presence of daidzein and p-coumaric acid in small concentration. [21]

In last few years few flavonoids and phenolic acids were identified in *Vigna radiata* seeds and sprouts. These compounds shown various biological activities. Identified flavonoids includes daidzein, ononin, formononetin, isoformononetin, 6, 7, 4′-trihydroxyisoflavone,genistin, prunetin, 2′-hydroxygenistein, apigenin, vitexin, isovitexin, rutin, quercetin-3-glucoside, kaempferol, kaempferitrin, neohesperidin, naringenin, delphinidin, etc. Few phenolic acids have been identified in seeds and sprouts of mung bean includes p-hydroxybenzoic, protocatechuic, syringic, gallic acid, vanillic acid, gentisic acid, shikimic acid, cinnamic acid, caffeic acid, chlorogenic acid etc. [9]

Results of present study demonstrated dose dependent decrease in cell viability of MCF-7 breast cancer cells in both MTT and SRB assays. It showed good cytotoxic properties. Extracted ion chromatogram of fractions shows presence of rutin, gallic acid, synergic acid, cinnamic acid, kaempferol and quercetin. Rutin has been extensively studied for anticancer potential. Rutin is effective against HL-60, SW480, LAn-

5, CRC, B16 melanoma cells, HTC hepatic cell lines. [22]Rutin inhibit cancer cell growth by cell cycle arrest and/or apoptosis, along with inhibition of proliferation, angiogenesis, and/or metastasis in colorectal cell lines[23]Quercetin, demonstrated inhibition of the proliferation of the ovarian cancer cell line OVCA 433 [24]Quercetin produced apoptosis and barred metastasis in pancreatic cancer[25]. Kaempferol has anti-inflammatory and antioxidant potential. Kaempferol possibly be involved in the regulationsof cell cycle, metastasis, angiogenesis and apoptosis in various cancer cell types. [26]

CONCLUSION

Reported literature suggest mung bean and udid bean plant usually possesses very high levels of antioxidants that are well known to act as potent anticancer and immunomodulatory agents. One of the possible mechanisms responsible for the cytotoxic effect of fractions of *Vigna mungo* and *Vigna radiata* is the presence of flavonoids and phenolics in isolated fractions which has a role in radical-mediated pathogenesis such as carcinogenesis.

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