

Antibacterial, Cytotoxicity Studies of *Azukia mungo* (L.) Masam and Correlation with *in silico* Docking Studies

K. Akilandeswari*, M. Vijayalaksmi, K. Kavitha, Girija M.

Department of Pharmaceutical Technology, Bharathidasan Institute of Technology, Anna University, Tiruchirappalli, Tamil Nadu, India

Abstract

Introduction and Background: *Azukia* genus plants rich in proteinaceous anti-nutrients like tannins (especially condensed tannins) has been shown to have anti-bacterial activity against *Staphylococcus aureus* and antineoplastic activity against lung and liver cancer cells. Five types of procyanidins (condensed tannins) have already been isolated from *Azukia mungo* and structurally elucidated. But its activity against the Methicillin resistant *Staphylococcus aureus* (MRSA) has not yet been shown. Hence we focus our work in exploring the antibacterial activity against MRSA strain along with the investigation of anticancer activity against HeLa cells. **Methods:** *Azukia mungo* seeds were collected and extracted. The extracted seeds were subjected to phytochemical screening to identify the chemical constituents. Qualitative identification of tannins in the extracts was performed through HPTLC using *n*-butanol: glacial acetic acid water as solvent system. The antibacterial and anticancer activity was predicted using flexible (GEM dock software) docking of procyanidins as ligands against several MRSA receptors and cervical cancer responsible receptors. The MTT assay was used to make an assessment of tumor-inhibitory action of *Azukia mungo* extract of acetone and water on HeLa cells. **Results and findings:** The various extracts of *Vigna mungo* were subjected to screening for its phytochemical constituents that showed the presence of specific constituents like alkaloids, tannins, flavonoids and steroids. In HPTLC, the extract peaks in the graph compared to the standard peak tannic acid were found to be 0.05 and 0.81 retention factors. Results of docking studies showed higher docking energy that implies good binding energy and hence more efficiency in blocking the activity of particular protein. The good binding energy of the ligand with active site of the receptor revealed -133.47 kcal/mol for MRSA receptor, and cancer receptor -108.45 kcal/mol. For MRSA and cervical cancer, maximum docking energy was exhibited between procyanidin A2 with 2YVW (penicillin binding protein receptor) and procyanidin B1 with HMG CoA reductase. This has been subsequently proved in the zone of inhibition of 27 and 17 mm, minimum inhibitory concentration of 62.5 and 125 µg/ml and in cytotoxicity studies, HeLa cell viability was reduced significantly in 24 h treatment. In 200 µg, percentage cell viability of acetone extract was 52.54% and in 250 µg, percentage cell viability of water extract was 48.66%. **Conclusion:** The increasingly widespread emergence of bacteria resistant to multiple antibiotics may be overcome partially by utilizing these natural compounds from *Azukia mungo* which was screened and its effectiveness against MRSA as alternative approaches, thereby reducing the additional usage of antibiotics. However, further improvements in the chemical structure of the drugs are needed to produce a newer class of drugs to treat MRSA infections and anti-cancer drugs.

Keywords: Phytochemical screening, *in silico* docking, antibacterial, cytotoxicity studies, *Azukia mungo*

*Author for Correspondence E-mail: akilaaaut@gmail.com

INTRODUCTION

The *Vigna mungo* belongs to the family *Fabaceae*. Black gram is used as diuretic, dropsy and cephalgia [1]. Clinical reports proved that effective components from *Vigna mungo* bean show anti anaphylaxis

property which can be used to treat hives [2].

The *Vigna* genus plants are rich in proteinaceous nutrients like tannins (especially condensed tannins). Presence of plant

constituents such as alkaloids, tannins, flavonoids and steroids in *Vigna mungo* were identified by phytochemical screening. Quantitative identification of tannins in *Vigna mungo* was performed through TLC and HPTLC [3]. Procyanidins have anti-neoplastic activity against lung and liver cancer. Procyanidins were isolated from *Pinus koraiensis* bark which has been investigated for its anti-cancerous activity against C14 cervical cancer cells.

On cervical cancer, the malignant neoplasm arising from cells originating from the cervix uteri in females has a death rate of more than 70% in most of the developed countries. Human papillomavirus (HPV) infection appears to be a necessary factor in the development of almost all cases (90%) of cervical cancer. On the other hand, procyanidins isolated from *Pinus koraiensis* bark has been investigated for its anticancerous activity against C14 cervical cancer cells [4]. Cytotoxicity study of *Vigna mungo* was used to make an assessment of tumor-inhibitory action. Procyanidins which were used as ligands against several cervical cancer responsible receptors are now predicted using flexible docking. Hence we focus our work with investigation of *Vigna mungo* against HeLa cells [5].

MATERIALS AND METHODS

Requirements

Vigna mungo seeds were collected from a shop in Trichy region. Authentication of these seeds was bought from St. Joseph College, Trichirappalli. Acetone, water, hydrochloric acid (HCl), Dragandroff's reagent, sulfuric acid, glacial acetic acid, sodium nitrite, gelatin, n-butanol, acetic anhydride were used.

Plant Collection and Extraction

The *Vigna mungo* seeds were immersed in water for four days (Maceration process) and filtered and the filtrate was kept in hot air oven at 50°C for 24 h to obtain the product after evaporation of water [6]. *Vigna mungo* powder was immersed in acetone and refluxed for 15 min and filtered. The filtrate was kept in the rotary vacuum evaporator to obtain the product after recovery of solvent [7].

Phytochemical Screening of the Extract

Phytochemical analysis for identification of composite chemical constituents of the extracts that include saponins, steroids, and tannins was carried out using following procedure.

Test for Carbohydrates

Dissolve a small quantity of the extract in 4 ml of distilled water and filtered. The filtrate was subjected to Molish's test for the presence of carbohydrate.

Molish's Test: 2 ml of extract and 1 ml of α -naphthol solution were added and concentrated sulphuric acid was added through the sides of the test tube. Formation of the violet color ring at the junction of two liquids indicated the presence of carbohydrates.

Test for Phytosterol

Liebermann-Burchard's Test: A small amount of the extract was dissolved in a few drops of glacial acetic acid. 3 ml of acetic anhydride was added, followed by adding few drops of concentrated sulfuric acid. The appearance of bluish green color shows the presence of phytosterol.

Salkowski Test: Small quantity of the extract was dissolved in chloroform and the resulting solution was then shaken with a few drops of concentrated sulfuric acid. The appearance of bluish green color shows the presence of phytosterol.

Tests for Tannins and Phenolic Compounds

A small quantity of the extract was dissolved in water, warmed and filtered. The resulting filtrate was used for the following tests:

- **Ferric Chloride Test:** A small amount of the filtrate was treated with a neutral ferric chloride solution. The appearance of violet color indicated the presence of phenols.
- **Gelatin Test:** A small amount of the filtrate was treated with a 1% w/v solution of gelatin in water containing 10% sodium chloride. The appearance of cream precipitate indicated the presence of phenolic compounds.
- **Lead Acetate Test:** A small amount of the filtrate was treated with 10% lead acetate solution. The appearance of white precipitate indicated the presence of phenolic compounds.

Test for Flavonoids

A small quantity of the extract was shaken with a few ml of water and the resulting mixture was subjected to the following tests:

- **Shinoda's Test:** A small quantity of test sample was dissolved in 5 ml of alcohol (95%) and treated with a few drops of concentrated hydrochloric acid and 0.5 g of magnesium turnings. Development of a pink color within a minute indicated the presence of flavonoids.
- **Fluorescence Test:** A few mg of the extract was dissolved in alcohol and a drop of the resulting solution was placed on Whatmann filter paper and observed under UV light. The appearance of fluorescence indicated the presence of flavonoids.

Tests for Proteins and Amino Acids

A small quantity of the extract was shaken with a few ml of water and the resulting mixture was subjected to the following tests:

- **Millon's Test:** When the test sample was treated with a Millon's reagent (Mercuric nitrate solution), formation of a white precipitate indicated the presence of proteins.
- **Ninhydrin Test:** When the test sample was treated with 0.1% w/v solution of ninhydrin in n-butanol, appearance of the violet or purple color indicated the presence of amino acid.
- **Biuret Test:** When the test sample was treated with equal volume of 5% sodium hydroxide solution and 1% copper sulphate reagent, the appearance of the pink to purple color indicated the presence of proteins and free amino acids.

Test for Lignin

A small quantity of the extract was treated with a few drops of phloroglucinol and

hydrochloric acid. The appearance of pink or red color indicated the presence of lignin [6, 7].

Culture Conditions: MRSA

MRSA cells grow well in ordinary media under aerobic conditions. Nutrient broth media is used for the growth of MRSA. 100 ml nutrient broth media (21 g/l) was prepared and sterilized. The isolated species were transferred into nutrient broth medium. The culture flask was then incubated for 24 h in an incubator at 37°C. After the incubation, the bacterial growth was identified by taking OD in spectrophotometer.

MTT assay

Cells were harvested and diluted. 1×10^4 cells/well were seeded into 96-well plates 100 µl of DMEM medium was added to well containing cells. The cells were allowed to adhere at an optimum condition (Overnight Incubation at 37°C in 5% CO₂ atmosphere). After overnight incubation, the culture medium was removed and cells were rinsed with phosphate buffered saline (1×PBS) and incubated with different concentrations of *Vigna mungo* water extract and acetone extract (50–500 µg) in complete DMEM medium for 24 h. After 24 h of treatment, 20 µl MTT (5 mg/ml in 1×PBS) was added to each well and incubated for an additional 4 h at 37°C to allow mitochondrial dehydrogenase to convert MTT into insoluble formazan crystals. The medium was then aspirated, and formazan was solubilized by adding 200 µl of DMSO. The absorption of solubilized formazan was measured at the wavelength of 590 nm with reference wavelength at 620 nm in a micro titer plate reader. The percent of inhibition of each concentration was calculated by following formula [8]:

$$\text{Percent of inhibition} = \frac{\text{Control optical density} - \text{Dose optical density} \times 100}{\text{Control optical density}}$$

GEMDOCK: A Generic Evolutionary Method for Molecular Docking

GEMDOCK is a program for computing a ligand conformation and orientation relative to the active site of target protein. The target PDB structure with the ID: 1R31, 2FK4, 2B9D were downloaded from PDB and the ligand structures from chemsketch were taken. The target was loaded into the GEMDOCK. The binding site was specified and prepared. The ligand compound folders were loaded and the compound was prepared. Population size was set to 50. Generations was set to 10. Number of solutions was set to 1. Then docking was started. The docked poses were analyzed after the completion of docking process [9].

RESULTS

The docked ligand molecules were selected based on docking energy and good interaction

with the active site residues. The Table 1 and Figures 1 and 2 give the values of the binding energies along with the Vander Waal forces [10]. The maximum docking energy was exhibited between procyanidin B1 with HMG CoA reductase [11].

The energy value is lesser than that for the already available drugs Bleomycin, cisplatin and topotecan as shown in Table 1. This positive result can be correlated to the optimized binding of procyanidin B1 to the active site of HMG CoA reductase [11]. Other combinations also gave significant interaction values. But the procyanidin C1 was incompatible with the docking with any of the receptors due to its higher energy when compared to other ligands. This implicates that procyanidin C1 can be less considered for the activity prediction [12].

Table 1: Results of the Docking Studies between the Types of Procyanidin and Various Receptors (1R31-HMG CoA Reductase, 2FK4-HPV16 E6 Cervical Oncoprotein and 2B9D-E7 Cervical Cancer Oncoprotein).

S. No.	Receptor	Ligand	Energy (kcal/mol)	van der Waals Force	Hydrogen Bond
1	1R31	Bleomycin	-18.11	-7.86	-10.25
2	1R31	Cisplatin	-23.55	-23.55	0
3	1R31	Topotecan	53.28	58.72	-5.44
4	1R31	Procyanidin A2	-73.76	-73.21	-0.56
5	1R31	Procyanidin B1	-108.45	-94.09	-14.36
6	1R31	Procyanidin B2	-97.87	-95.80	-2.07
7	1R31	Procyanidin B5	-77.44	-47.32	-30.12
8	1R31	Procyanidin C1	101.59	109.47	-7.87
9	2B9D	Bleomycin	-66.34	-59.36	-6.97
10	2B9D	Cisplatin	-21.99	-21.99	0
11	2B9D	Topotecan	263.8	278.1	-14.3
12	2B9D	Procyanidin A2	-61.21	-54.86	-6.35
13	2B9D	Procyanidin B1	-49.97	-49.97	0
14	2B9D	Procyanidin B2	-68.98	-60.48	-8.50
15	2B9D	Procyanidin B5	-73.24	-68.65	-4.59
16	2B9D	Procyanidin C1	33.58	46.58	-12.99
17	2FK4	Bleomycin	-65.92	-54.31	-11.61
18	2FK4	Cisplatin	-22.92	-22.92	0
19	2FK4	Topotecan	190.72	188.91	1.81
20	2FK4	Procyanidin A2	-69.41	-55.21	-14.20
21	2FK4	Procyanidin B1	-77.73	-67.44	-10.30
22	2FK4	Procyanidin B2	-71.86	-69.22	-2.63
23	2FK4	Procyanidin B5	-93.3	-71.51	-21.79
24	2FK4	Procyanidin C1	15.97	25.18	-9.21

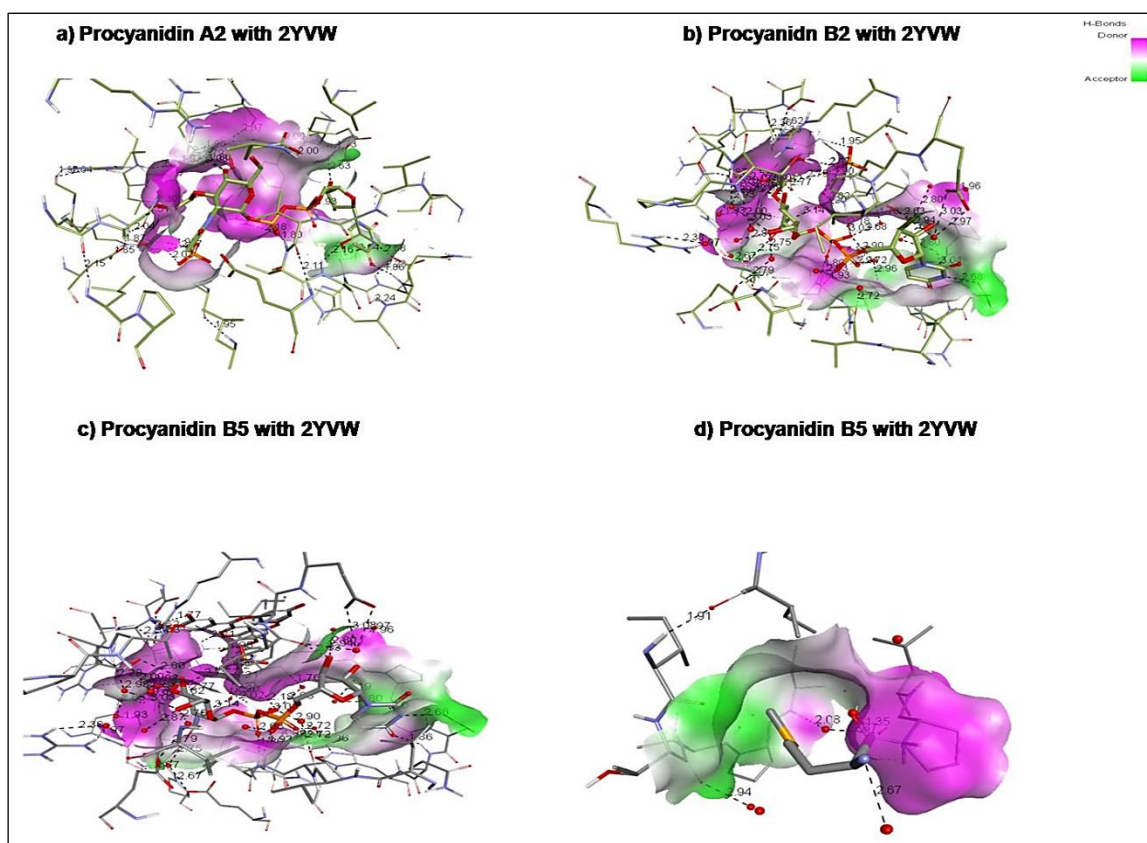


Fig. 1: Individual Docked Poses for the Procyanidin with Different Receptors.

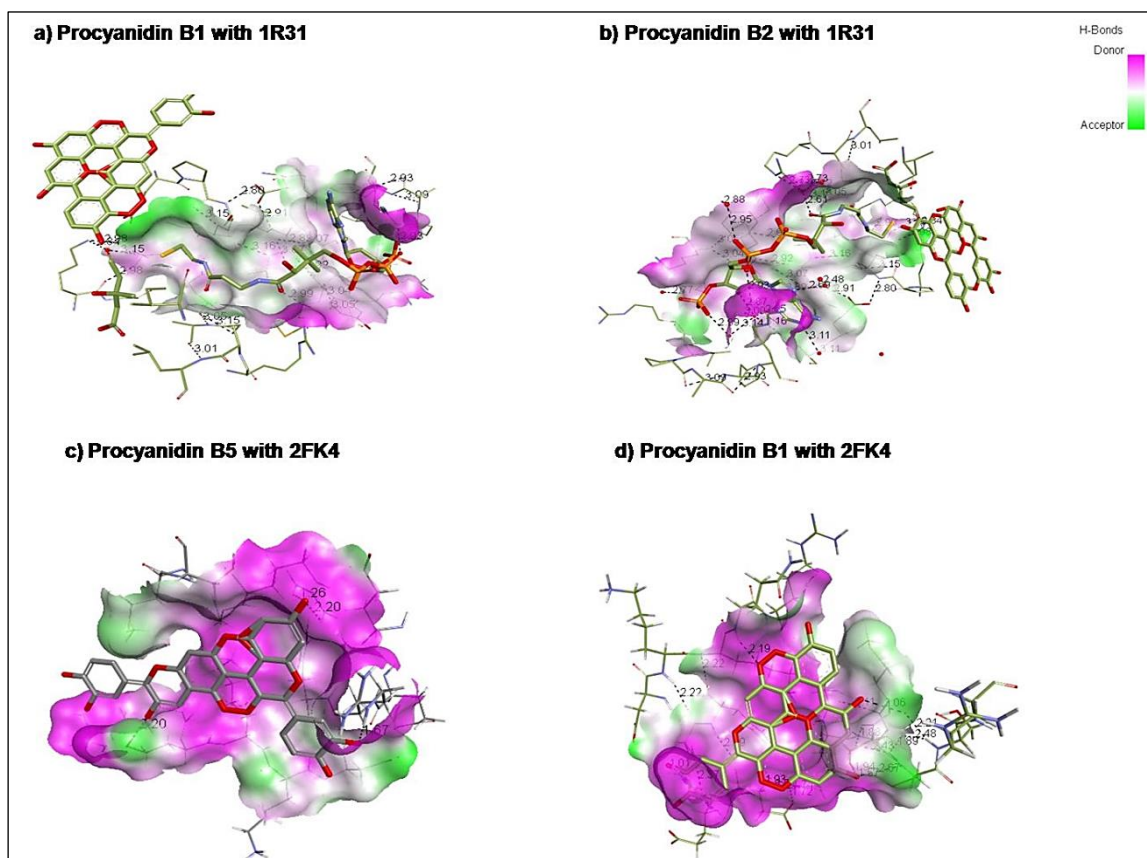


Fig. 2: Individual Docked Poses for the Procyanidin with Different Receptors.

The condensed extracts which were obtained after evaporation of the solvent were found to be 30 and 40% yield for acetone and water respectively, calculated from the formula:

$$\text{Percentage yield} = (\text{Weight of dried extract} / \text{Weight of the sample taken}) \times 100$$

The above extracts were subjected for phytochemical screening for the presence of alkaloids, tannins, flavonoids, saponins, terpenoids, glycosides, reducing sugars, and steroids. Their respective presence or absence of the separate constituents is shown in the Table 2. Both the extracts also gave positive result for the presence of condensed tannins [13].

Qualitative identification of tannins in the extracts was performed through HPTLC method using n-butanol: glacial acetic acid: water as 4:2:1 solvent system. The peaks for extracts in the graph (Track 2, 3 for acetone extract and track 4, 5 for water extract) in

reference to the standard peak for the tannic acid (Track 1, 2) were found in accordance with respect to their retention factors at 0.05 and 0.81 (Figures 3 and 4).

Table 2: Phytochemical Screening of Acetone and Water Extracts of *Vigna mungo*.

Name of the Constituent	Acetone Extract	Water Extract
Alkaloids	+	+
Tannins	+	+
Flavonoids	+	+
Saponins	-	-
Terpenoids	-	-
Glycosides	-	-
Reducing sugars	-	-
Steroids	+	+

However the widened area of the peaks of the extracts indicates the presence of several types of tannins say, the condensed and hydrolysable tannins as they are crude extracts [8].

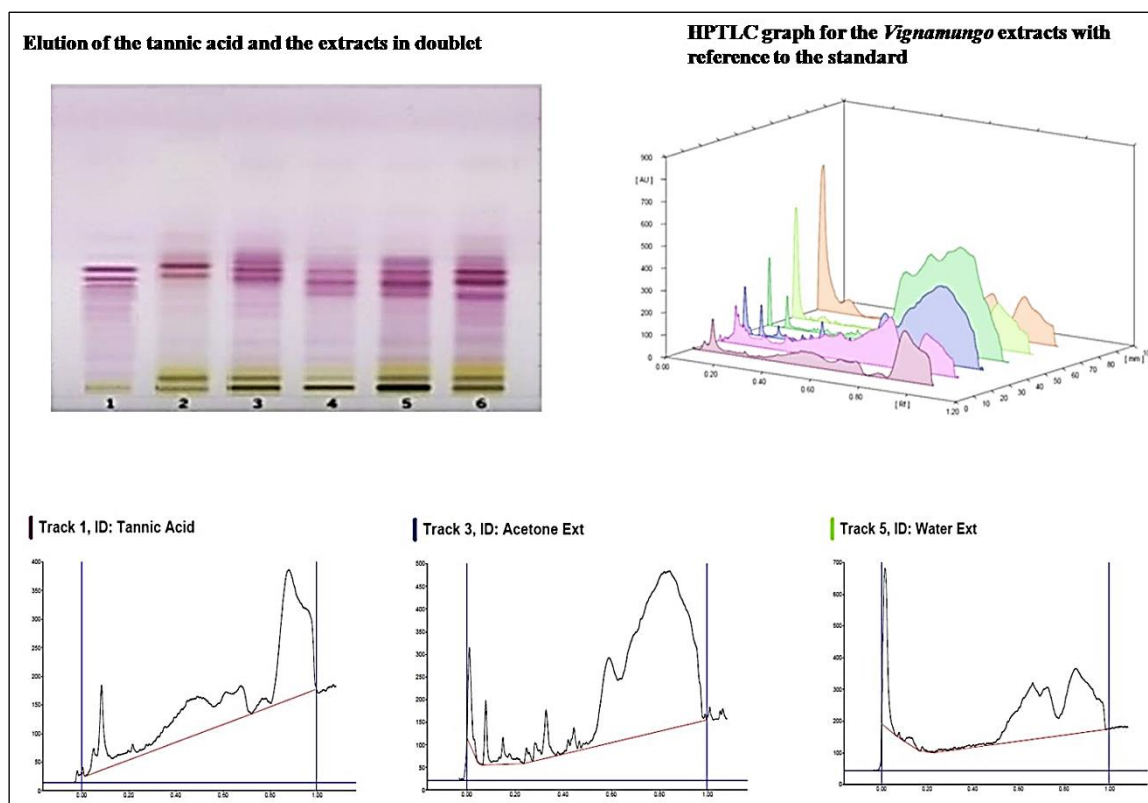


Fig. 3: HPTLC Graph for the *Vignamungo* Extracts with Reference to the Standard.



Fig. 4: Antibacterial Activity of Vignamungo Extract against MRSA Showing Zones of Inhibition.

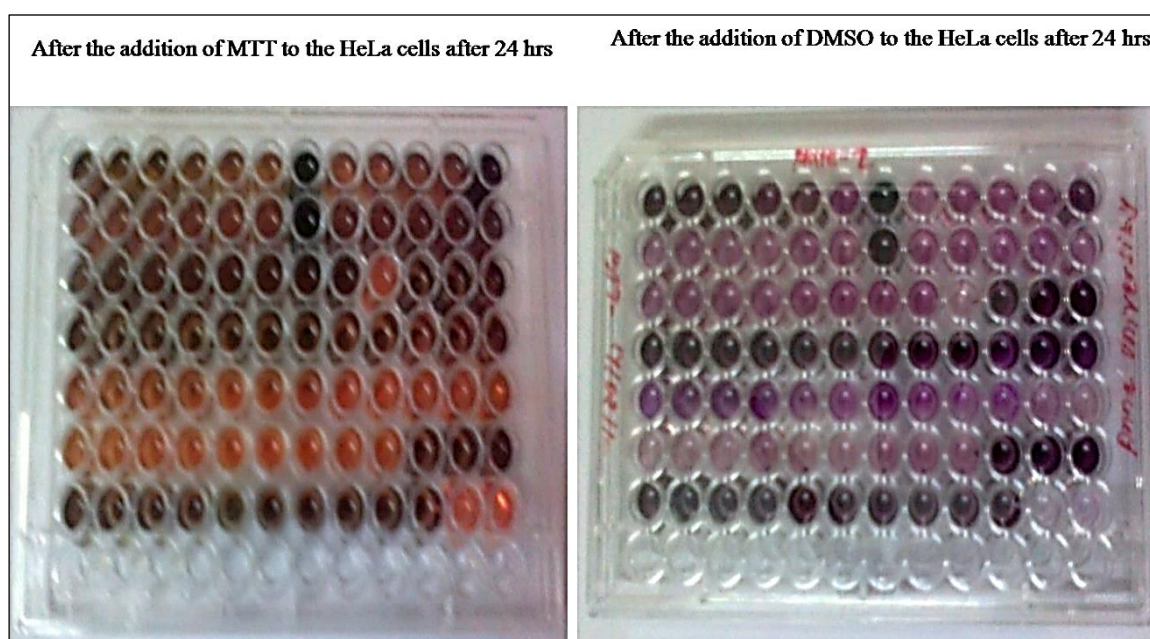


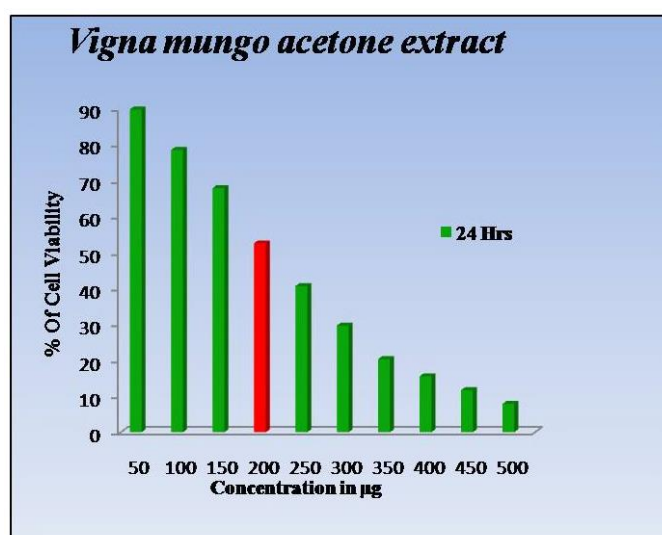
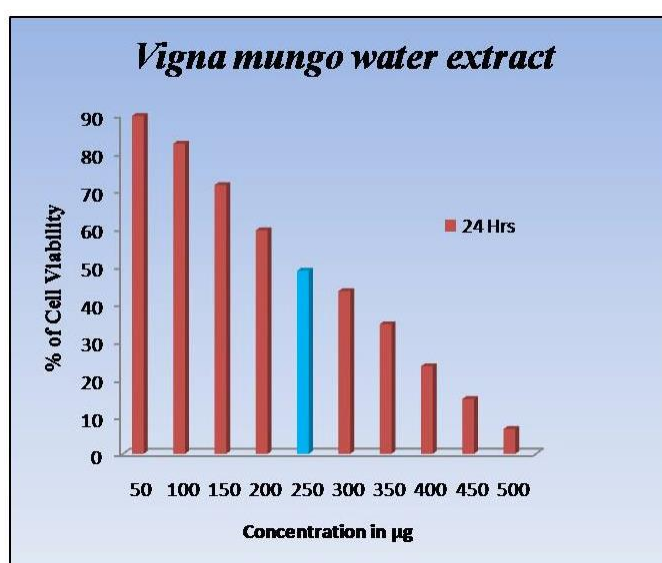
Fig. 5: After the Addition of MTT to the HeLa Cells after 24 h.

The MTT assay was used to make a preliminary assessment of the tumour-inhibitory action of *Vigna mungo* acetone and water extract on HeLa cells, to determine the significant IC_{50} values at the specific time points (Figures 6 and 7). HeLa cell viability was reduced significantly in 24 h treatment; in 200 and 250 μg concentrations of *Vigna mungo* acetone and water extract, 52.54 and 48.66% 24 h treatments, respectively. Percentage of cell survival was reduced

gradually with increase in the *Vigna mungo* acetone and water extract treatment concentration. From these results, the IC_{50} value of *Vigna mungo* acetone and water extract were calculated as =200 and 250 μg . In the present study, we found that *Vigna mungo* acetone and water extract inhibit the growth of HeLa cells by decrease in cell viability determined by MTT assay (Figure 5 and Table 3)) [14–22].

Table 3: Decrease in Cell Viability of HeLa Cells Caused by *Vigna mungo* Acetone and Water Extract.

Concentration ($\mu\text{g/ml}$)	% of Cell Viability for Acetone Extract	% of Cell Viability for Water Extract
50	89.81	89.81
100	78.54	82.43
150	67.91	71.45
200	52.54	59.44
250	40.65	48.66
300	29.66	43.24
350	20.34	34.42
400	15.56	23.25
450	11.7	14.55
500	7.86	6.6

**Fig. 6:** Exposure of HeLa Cells to Various Concentrations of *Vigna mungo* Acetone Extracts.**Fig. 7:** Exposure of HeLa Cells to Various Concentrations of *Vigna mungo* Water Extracts.

Cell viability was analyzed by MTT assay. Dose dependent inhibition of cell growth was observed after 24 h [23–38].

DISCUSSION

The present extraction work has yielded a comparatively good yield of 30% for water extract and a relatively higher yield of 40% for acetone extract where the yield of tannins using the solvent as water has given a maximum of 32%. Identification of the presence of tannin in the extracts was confirmed through the HPTLC method. The peaks for the extracts were in accordance with the reference standard, tannic acid at their retention factors. The agar plate method showed a higher inhibition of 27 mm and the least inhibition of 12 mm. The acetone extract has given the higher zone of inhibition compared to the water extract. This complies with the docking study values when done with the procyanidins against several receptors. When the compounds were docked against the cervical cancer receptors, procyanidin B1 exhibited stronger interaction with the potential target of the cervical cancer, HMG CoA reductase than the FDA approved drugs cisplatin, bleomycin and topotecan. Hence the extracted compounds Procyanidin A2, B1, B2 and B5 (except procyanidin C1) seem to act as potent inhibitors of those receptors. However, further pharmacological studies and improvisation of the drug properties are needed to be carried out for the confirmation of the activity and could act as a new class of drugs to treat cervical cancer [29–31].

CONCLUSION

This study concluded that computer aided screening is an effective alternative for preliminary identification of novel remedies and in reducing many complexities of drug discovery process. Natural compound from *Vigna mungo* was screened and its effectiveness against cervical cancer are done and compared with known marketed drugs. All the results gave good correlation with the expected outcome. On the other hand, it forms a natural remedy for the curing of cervical cancer also. Thus natural products serve a

good alternative for the therapy of diseases of mankind from traditional times till date.

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