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ANTIMICROBIAL, ANTIOXIDANT AND ANTICANER ACTIVITY OF FLAXSEED

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ABSTRACT

The present study was carried out to evaluate the Antimicrobial, Antioxidant, Cytotoxicity and Anticancer activity of Flaxseed extract. The two different solvents like water and ethanol were used for extraction. Antimicrobial activity was determined by Agar disc diffusion method and MIC. Aqueous and Ethanol extract of Flaxseed showed inhibition against *Salmonella typhi* and *Pseudomonas aeruginosa* and no inhibition effect was noticed against against *Escherichia coli, Shigella species, Proteus species* and *Staphylococcus aureus*. The flaxseed extract did not showed antifungal activity against the tested fungi –*Aspergillus niger, Aspergillus flavus, Rhizopus species, Penicillum species and Candida albicans.* Antioxidant activity was determined by DPPH and FRAP assay. Cytotoxicity was done on vero cell line and toxic free concentrations were estimated. Anticancer activity was done against HeLa cell lines by MTT assay. Cytotoxicity revealed that the flaxseed extracts were non-toxic and were capable of inhibiting the proliferation of HeLa cell line. Flax seed was found to contain Antibacterial, Antioxidant and Anticancer properties.

KEYWORDS: FLAXSEED, Antioxidant activity, DPPH assay, FRAP assay, MTT assay, HeLa cell line.

1.0 INTRODUCTION

Flaxseed is one of the oldest crops, have been cultivated since the beginning of civilization. The Latin name of flaxseed is *Linum ustatissimum* which means –very useful. They

belong to the family *Linaceae*. Humans have been eating flaxseed for thousands of years.[1]

Linseed has rich source of medicinal use, its major effects being as a laxative and expectorant that soothes irritated tissues, controls coughing and relieves pain in addition of being analgesic, demulcent, emollient, pectoral and resolvent.[2] There has been growing interest in the probiotic properties of flax and it is used to treat coronary heart disease, different types of cancer and neurological and hormonal disorders.[3] Flaxseed contains 40-50% of oil, 23-34% of protein, 4% of ash 5% of mucilage, 0.9-3% lignin precursors.[4]

Flaxseed has potential health benefits besides the nutrition, due to mainly three reasons: first due to its high content of ὼ-3 fatty acid; α- linolenic acid; second being rich in dietary soluble and insoluble fibers and third, due to its high content of lignans, acting as anti-oxidants and phytoestrogens.[5]

Flaxseeds are rich in secoisolariciresinoldiglucoside (SDG), the precursor of lignans, which show effective action on human health. SDG has a power to exhibit anticancer properties by inhibiting proliferation and growth. The nutraceutical effect of flaxseed has attracted researchers in recent years, leading to identification and isolation of various bioactive compounds.

In recent years, plant-based compounds are used in food and medical industries because of their high potential health benefits. It is well known that plants are the richest source of antioxidants and phenolic acids occurring in plants, primarily bound form as conjugates, with sugars, fatty acids, or protein act as an effective natural antioxidants.[6]

Many cancer patients seek treatments with complementary or alternative medicine (CAM). This is because the patient developed drug resistance towards chemotherapies. Many cancer patients don't tell the physician about usage of CAM. The increasing incidence of cancer all over the world urges to find out newer anticancer substances that could kill cancer cells without any side effects unlike chemotherapies.

2.0 METHODOLOGY

2.1 Plant Material

Linum usitatissimum (flaxseed) was purchased from the local super market in Anna nagar.

2.2 Extraction of Flaxseed

2.2.1 Ethanolic extraction

25 g of dried and ground flaxseed was added in 250 ml of the non- polar solvent, ethanol and the extract was carried out for 8hrs using Soxhelt apparatus followed by removal of solvent using rotary vacuum evaporator. [7]

2.2.2 Aqueous extraction

20 g of dried and ground flaxseed was added in 100 ml of the water and kept in a shaker with continuous agitation at 150rev/min for complete elucidation of active materials to dissolve in water. The extract was filtered using muslin cloth, followed by Whatman no.1 filter paper. The solvent was removed using a rotary evaporator. And the extract was stored in sterile glass bottles at room temperature until screened.

2.3 Antibacterial Activity Assay

2.3.1 Preparation of inoculum

The inoculum was prepared for each bacterium by using the direct colony suspension procedure (CLSI). The inoculum density was standardized using a barium sulphate turbidity standard, equivalent to 0.5 Mcfarland standards. The absorbance was measured using a spectrophotometer at 625 nm and it should be 0.08 -0.13 for the 0.5 Mcfarland standard.

2.3.2 Disc diffusion test

The antibacterial activity of flaxseed extracts were determined by standard disc diffusion method.[8, 9] Mueller-hinton agar was prepared, autoclaved and cooled medium was added to sterile petri dishes to give a uniform depth of 4mm and allowed to set. Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension and pressed firmly on the inside wall of tube to remove excess inoculum from the swab. The swab was streaked over the entire sterile agar surface. 1 mg/ml of seed extract was prepared for each solvent as the stock solution. The different concentrations (1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, and 62.5µg/ml) of the extract were prepared by double dilution method. The sterile filter paper discs were soaked in 15µl of each prepared concentration of the extract. Each disc was pressed down to ensure complete contact with the agar surface. Ampicillin was used as positive control. The plates were incubated at 37˚C for 16-18 hrs and the diameter of the zone of inhibition was measured around the discs.

2.4 Antifungal Activity

The inoculum was prepared for each fungus using Sabouraud Dextrose broth and incubated for 48 hrs at room temperature. The assay was performed by agar disc diffusion method. Sabouraud Dextrose Agar was prepared, autoclaved and the cooled medium was added to sterile petri dishes and allowed to set. A sterile cotton swab was dipped into the inoculum tubes and streaked evenly on the agar surface. The stock was prepared for 1mg/ml as stock solution and different concentrations (1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, and 62.5µg/ml) were obtained by double dilution method. The sterile discs were soaked in 15µl of each concentration and the plates were incubated for 24hrs and the diameter of the zone of inhibition was measured.

2.5 Minimum Inhibitory Concentration (MIC)

The antibacterial activity of flaxseed was determined by MIC method.

2.5.1 Preparation of inoculum

The inoculum was prepared by making a direct broth suspension from an isolated colony selected from 18 to 24 hrs agar plates. The turbidity was adjusted to 0.5 Mcfarland's turbidity standard.

2.5.2 Inoculation and incubation

Within 15 min after the inoculums were adjusted, 1 ml of inoculum should be transferred to tubes containing 1 ml of antimicrobial agents (flaxseed extracts) in dilution series. The tubes were incubated at 35±2˚C for 16 to 24 hrs. The amount of growth in tubes was compared with control tubes. The O.D values were taken at 517 nm and the recovery plates were plated for inhibiton of organism by the extracts.

2.6. Phytochemical Analysis[10]

2.6.1. Test for tannins

1 ml of the flaxseed extract was taken and added with few drops of 0.1% ferric chloride and observed for brownish green or blue-black coloration.

2.6.2. Test for saponins

A small amount of extract was shaken with little quantity of water. The suspension was shaken in a graduated cylinder for 15 min and observed for foam formation.

2.6.3. Test for flavonoids

1 ml of the sample was taken and added with concentrated hydrochloric acid and magnesium chloride and observed for tomato red color.

2.6.4. Test for alkaloids

1 ml of the sample was taken and added with few drops of Drangandoff reagent and observed for prominent yellow precipitate.

2.6.5. Test for protein

1 ml of the sample was added with few drops of Millon's reagent and observed for white precipitates.

2.6.6. Test for steroids

1 ml of the sample was mixed with two drops of 10% concentrated sulphuric acid and observed for brown color.

2.6.7. Test for anthraquinones

1ml of the sample was taken and added with few drops of aqueous ammonia and observed for change in color. Pink, red, or violet color in aqueous layer indicates the presence of anthraquinones.

2.6.8. Test for phenol

1ml of the sample was taken and 3 ml of 10% lead acetate solution was added and observed for a bulky white precipitate for the presence of phenolic compounds.

2.7.0 Antioxidant Assay

2.7.1 DPPH assay

DPPH assay was performed to determine the antioxidant activity of the aqueous and ethanolic extract of flaxseed.[11] DPPH (1,1-diphenyl-2-picrylhydrazyl) is characterised as a stable free radical. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Aliquot 3.7 ml of absolute methanol in all test tubes and 3.8ml of absolute methanol was added to blank. Add 100µl of BHT to tube marked as standard and 100µl of respective samples to all other tubes marked as tests. 200µl of DPPH reagent was added to all the test tubes including blank. Incubate all test tubes at room temperature in dark condition

for 30 minutes. The absorbance of all samples was read at 517 nm.

Calculation

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test}) \times 100}{(\text{Absorbance at blank})}$$

2.8.0 Ferric Ion Reduction Potential Assay

FRAP assay is a novel method for assessing – Antioxidant power. Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentrations.[12]

2.8.1 FRAP Assay Procedure

1ml of distilled water and 80µl of test sample was pipetted into the standard 4ml plastic cuvette. 600µl of incubated FRAP Reagent was added to the cuvette, which was briefly inverted to mix the solutions. The reagent blank was also prepared and 80µl of distilled water was added instead of the test sample. Change in absorbance at 593 nm (as a result of reduction of the Fe^{2+} -TPTZ complex at low pH) was recorded exactly at 4 minutes using spectrophotometer. Each sample dilution was tested in triplicate to allow a mean absorbance to be calculated.

2.9.0 Cytotoxicity Activity

2.9.1 Cell line and culture

Vero cell line was obtained from National Centre for cell sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml) CO_2 at 37˚C.

2.9.2 *In vitro* assay for cytotoxicity activity (MTT assay)

Cells (1×10^5/well) were plated in 24-well plates and incubated at 37˚C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 hrs. After incubation, the samples were removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100 µl/well (5mg/ml) of 0.5% 3-(4,5- dimethly-2-thiazolyl)-2,5-diphenyl—tetrazolium bromide (MTT) was added and incubated for 4 hrs. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV- Spectrophtometer using DMSO

as the blank. Measurements were taken and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula.

$$\% \text{ Cell viability} = \frac{A_{570} \text{ of treated cells} \times 100}{A_{570} \text{ of control cell}}$$

Graphs were plotted using the % of cell viability at Y-axis and concentration of the sample in X- axis. Cell control and sample control are included in each assay to compare the full cell viability assessments.[13]

2.10 ANTI-CANCER ACTIVITY

2.10.1 Cell line and culture

HeLa cell line was obtained from National Centre for cell sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml) CO_2 at 37˚C.

4.10.2 *In vitro* assay for anti-cancer activity (MTT assay)

CELLS (1×10^5 /well) were plated in 24-well plates and incubated at 37˚C with 5% co_2 condition. After the cell reaches the confluence, the various concentration of the sample was added and incubated at 37˚C with 5% co_2 for 24 hrs. After incubation the sample was removed from the well and washed with phosphate buffer saline (pH 7.4) or DMEM without serum 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethly-2-thiozolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 hrs. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV Spectrophotometer using DMSO as blank. Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % of cell viability was calculated using the following formula,

$$\% \text{ Cell viability} = \frac{A_{570} \text{ of treated cells} \times 100}{A_{570} \text{ of control cells}}$$

Graphs are plotted using the % of the cell viability at Y-axis and the concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability.

3.0 RESULTS

Linum usitatismum (Flaxseed)

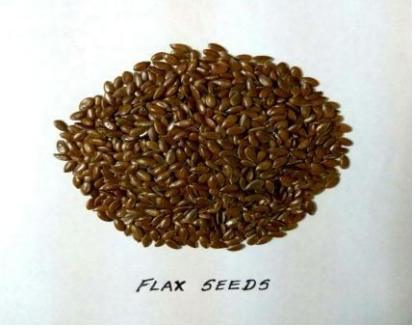


Fig 1: Flaxseed.

Fig 2: Flaxseed powder.

Fig 3: Antibacterial activity of aqueous extract of Flax seed against *Salmonella typhi.*

Fig 4: Antibacterial activity of aqueous extract of Flax seed against *Pseudomonas aeruginosa.*

Table 1: Antibacterial assay of aqueous extract of flax seed.

Organism	Zone of inhibition (mm) Concentration (µg/ml)					Antibiotics (1mg/ml) Ampicillin
	1000	500	250	125	62.5	
Salmonella typhi	15	10	5	NIL	NIL	25
Shigella	NIL	NIL	NIL	NIL	NIL	15
Pseudomonas aeruginosa	16	12	6	NIL	NIL	25
Proteus species	NIL	NIL	NIL	NIL	NIL	18
Staphylococcus aureus	NIL	NIL	NIL	NIL	NIL	15
Escherichia coli	NIL	NIL	NIL	NIL	NIL	18

Table 2: Antibacterial activity of Ethanol extract of Flaxseed.

Organism	Zone of inhibition (mm) Concentration µg/ml					Antibiotics (1mg/ml) Ampicillin
	1000	500	250	125	62.5	
Salmonella typhi	10	8	5	NIL	NIL	24
Shigella species	NIL	NIL	NIL	NIL	NIL	14
Pseudomonas aeruginosa	12	9	5	NIL	NIL	20
Proteus species	NIL	NIL	NIL	NIL	NIL	15
Staphylococcus aureus	NIL	NIL	NIL	NIL	NIL	18
Escherichia coli	NIL	NIL	NIL	NIL	NIL	18

Fig 5: Antibacterial activity of Ethanol extract of flaxseed against *Salmonella typhi.*

Fig 6: Antibacterial activity of Ethanol extract of flaxseed against *Pseudomonas aeruginosa.*

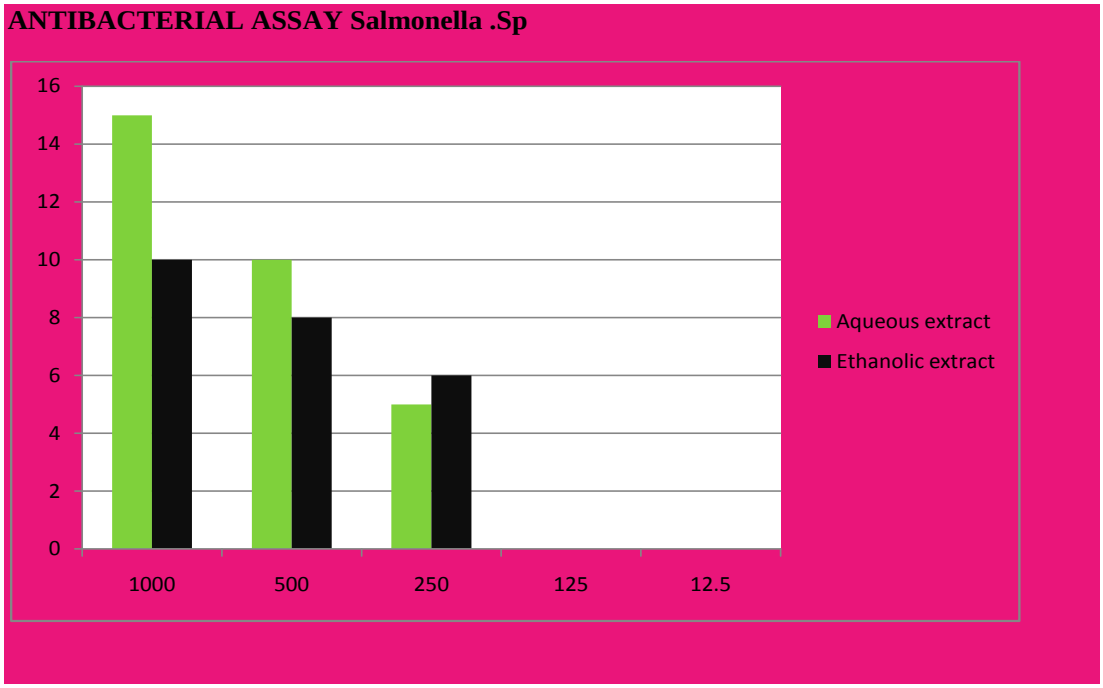


Fig 7: Antibacterial assay of aqueous and ethanol extract of *Salmonella typhi*.

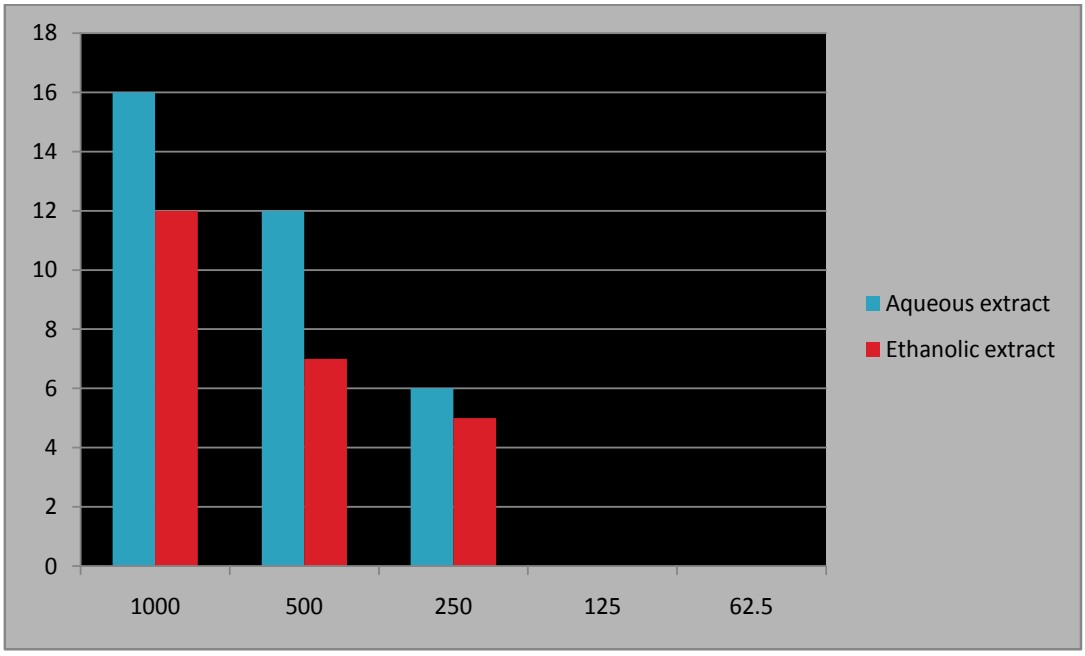


Fig 8: Antibacterial assay of aqueous and ethanol extract against *Pseudomonas aeruginosa*.

Table 3: Minimum Inhibitory Concentration of Aqueous extract of Flaxseed.

Concentration (µg/ml)	*Salmonella typhi*	*Pseudomonas aeruginosa*
1000	0.016	0.003
500	0.164	0.038
250	0.505	0.130
125	0.697	1.119
62.5	1.165	1.290
31.2	1.215	1.385
15.5	1.230	1.419
7.8	1.336	1.446

Table 4: Minimum Inhibitory Concentration (MIC) of Ethanol extract of flaxseed.

Concentration (µg/ml)	*Salmonella typhi*	*Pseudomonas aeruginosa*
1000	0.076	0.016
500	0.112	0.119
250	0.150	0.722
125	0.770	1.353
62.5	1.179	1.388
31.2	1.184	1.418
15.5	1.258	1.422
7.8	1.266	1.473

Table 5: Antioxidant activity of flaxseed by DPPH Assay method.

S.NO	Sample	Concentration (µg/ml)	O. D	DPPH activity
1	Flaxseed aqueous	1000	0.003	88.88%
2	Flaxseed ethanol	1000	0.008	70.37%

CONTROL O.D: 0.02

Table 6: Antioxidant activity of the Flaxseed using FRAP assay method.

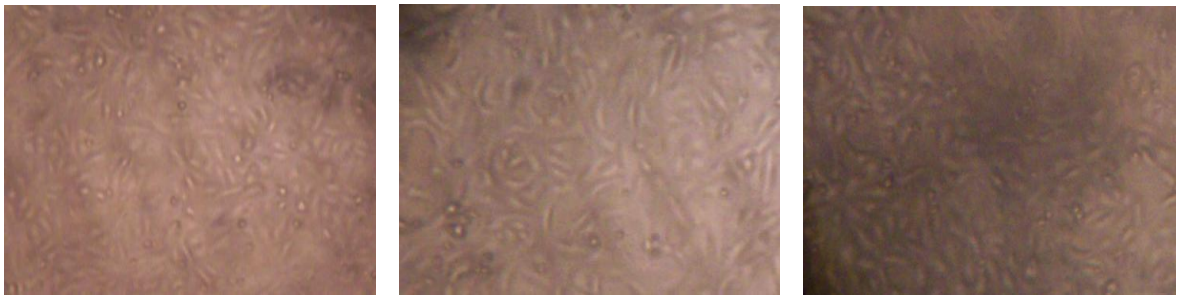
S. No	Sample	O.D Value for sample at 0	0 to 4 min Name of the sample	FRAP (µm)
1	Flaxseed aqueous	0.081	0.160	395µm
2	Flaxseed ethanol	0.282	0.401	595µm

O.D value for ascorbic acid at 0 TO 4 MIN= 0.2

Table 7: Cytotoxicity effect of ethanol extract of flaxseed *on Vero* cell line.

S. No	Concentration (µg/ml)	Dilutions	Absorbance	Cell viability (%)
1	1000	Neat	1.900	170.55
2	500	1:1	1.933	173.51
3	250	1:2	1.973	177.10
4	125	1:4	2.045	183.57
5	62.5	1:8	2.096	188.15

6	31.2	1:16	2.160	193.89
7	15.6	1:32	2.233	200.44
8	7.8	1:64	2.287	205.29
9	Cell control	-	1.114	100

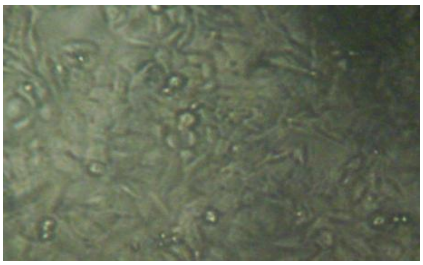


Normal Vero Cell line **Toxicity - 1000µg/ml** **Toxicity-62.5µg/ml**

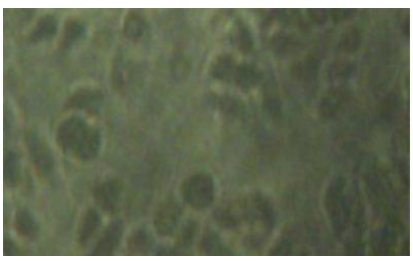
Fig 9: Cytotoxicity effect of ethanol extract of flax seed on Vero cell line.

Table 8: Anticancer effect of ethanol extract of flaxseed *on HeLa* cell line.

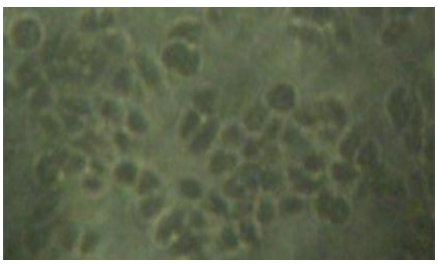
S. No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.225	24.67
2	500	1:1	0.271	29.71
3	520	1:2	0.332	36.40
4	125	1:4	0.398	43.64
5	62.5	1:8	0.464	50.87
6	31.2	1:16	0.514	56.35
7	15.6	1:32	0.582	63.81
8	7.8	1:64	0.642	70.39
9	Cell control	-	0.912	100



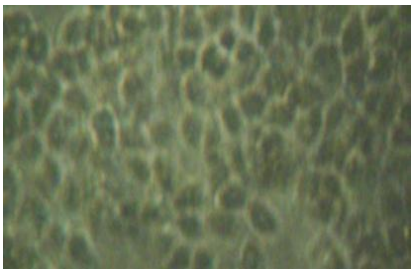
Normal HeLa cell line



Toxicity-1000µg/ml



Toxicity-62.5µg/ml



Toxicity- 7.8µg/ml

Fig 10: Anticancer effect of ethanol extract of flaxseed on *HeLa cell* line.

4.0 DISCUSSION

In the present study, Aqueous and ethanol extracts of flaxseed were tested for Antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Shigella species, and Proteus Species* and *Staphylococcus aureus*. The Antifungal activity was tested against *Aspergillus niger, Aspergillus flavus, Penicillium species, Rhizopus species and Candida albicans.* The Antimicrobial activity was determined by Agar Disc Diffusion method and *Minimum* Inhibition concentration (MIC). Aqueous and ethanolic extracts of flaxseed inhibited *Salmonella typhi* and *Pseudomonas aeruginosa* and no inhibition against *Escherichia coli, Shigella species, Proteus species* and *Staphylococcus aureus.*

Aqueous extract of flaxseed inhibited *Salmonella typhi* at two different concentrations - 1000µg/ml and 500µg/ml with zone of inhibition diameter -15 mm and 10 mm and inhibited Pseudomonas aeruginosa at the same concentration with a zone diameter -16 mm and 12 mm. (Refer fig 3, 4 & table 1). Ethanol extract of flaxseed also inhibited *Salmonella typhi* at two different concentrations 1000µg/ml and 500µg/ml with zone of inhibition diameter – 10 mm and 8 mm and inhibited *Pseudomonas aeruginosa* at the same concentration with a zone diameter-12 mm and 9 mm. (Refer fig 5, 6 & table 2).

The Minimum Inhibition Concentration of both aqueous and ethanol extracts were determined for *Salmonella typhi* and *Pseudomonas aeruginosa* and were found to be 500µg/ml for both. (Refer table 3 & 4) Aqueous and ethanol extract of flaxseed did not inhibit *Escherichia coli* which is a normal flora of human in the intestine rather it inhibited *Salmonella typhi* which cause typhoid and *Pseudomonas aeruginosa* which cause wound infection and could be used to treat the bacterial infections.

The Phytochemical screening revealed the presence of phenols and flavonoids. Flavonoids are the most commonly known for their antioxidant activity and act as transformers which

modify the body's reactions to carcinogens, viruses, and allergens.[6]

The stable compound DPPH gets reduced by gaining a hydrogen or electron and the violet color of the DPPH radical was reduced to yellow color. The percentage scavenging of DPPH radical for aqueous and ethanol extract was found to be 88.88% and 70.37% respectively. (Refer table 5) The change in color indicates that the extract was found to contain an antioxidant. The percentage scavenging of DPPH radical by ethanol extract of flax seed was found to be 80% at the concentration 500µg/ml.[14] Total antioxidant activity (TAA) were also determined using the ferric reducing of which is based upon reduction of Fe^{3+}-TPTZ complex under acidic condition. The increase in absorbance of blue colored ferrous form (Fe^{2+}- TPTZ complex) was measured at 593nm, flaxseed ethanol extract was found to have 595µm and aqueous extract - 395µm (Refer table 6).

Earlier reports on antioxidant activity of flaxseed are very rare in the literature. Therefore, it is very difficult to compare our results with that of previous studies. The flaxseed cake extracts contain phenolic compounds, including lignans, recommends them as multi-functional antioxidant and antibacterial additives for food products and cosmetics.[15] Estimation of in vitro maximal cytotoxic free concentration of the flax seed extract was done on Vero cell line and was found to be nontoxic from 1000µg/ml and was recorded as micrograph (Refer Table 7 & Fig 9).

The extract showed anticancer effect till the concentration of 62.5µg/ml and showed a potent activity against HeLa cell line. The effect of flaxseed extract on HeLa cell line at different concentration was recorded as micrograph (Table 8 & Fig 10). The concentration required for a 50% inhibition (IC_{50}) was determined as 62.5µg/ml. Flax seed has a broad spectrum anticancer activity. Linoorbitides (LOB) are one of the compound in flaxseed that has implications for anticancer and antioxidant activity. LOB3 inhibited Human melanoma cell line A375 and breast cancer cell lines Sk-Br-3 and proved to have highest in vitro antitumor activity.[16] Flaxseed inhibited the growth of human estrogen-dependent breast cancer and strengthened the tumor inhibitory effect of TAM (Tamoxifen) at both low and high E2 (17β-estradiol) levels.[17]

5.0 CONCLUSION

The flaxseed extracts exhibited antibacterial activity against *Salmonella typhi* and *Pseudomonas aeruginosa*. The aqueous extract showed better inhibitory activity when

compared with ethanol extract of flaxseed and did not possess antifungal activity against the tested fungi. Flaxseed extracts were found to possess a high antioxidant activity. The consumption of flaxseed may play a role in preventing human diseases such as Cancer, cardiovascular disease and aging. Flaxseed is proved be toxic free by testing the level of toxicity on Vero cell line. Anticancer activity on HeLa cell line shows that it has good anticancer activity at 62.5 µg/ml concentration. Flaxseed is effective in combating cancers and holds greater therapeutic potential for its application as a nutraceutical for the prevention and treating of cancer. Awareness must be created among people about the beneficial effects of flaxseed to intake in right proportion and thereby to prevent and reduce the risk of cancer as it is also found to be a good antioxidant and anticancer.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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