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RESEARCH ARTICLE

INVITRO CYTOTOXIC EVALUATION OF Annona Muricata (GRAVIOLA) LEAVES ON MCF-7 CELL LINE

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ABSTRACT

Annona muricata is a tropical plant which has been utilized as a folk medicine to treat many diseases including cancer and many other bioactivities. The aim of this study was to evaluate the phytochemicals present in the leaf extracts and to analyze its medicinal importance in cancer therapy. Additionally, the study determined the mineral elements such as Fe and Mg which are the structural component of tissues and essential for the functioning of all the cells. Vitamin C is a well-known antioxidant that helps to strengthen immune system by reducing the amount of free radicals in the body. *A. muricata* leaf extracts were evaluated for the scavenging assay (DPPH;1-1-diphenyl-2-picryhydrazyl), (FRAP-Ferric Reducing Antioxidant Power) and the study shows that the leaf extracts have good scavenging activity. The antibacterial activity of leaf extract was examined by disc diffusion method in Gram negative and Gram-positive bacterium. Invitro test against breast cancer cellgrowth was conducted using the MCF-7 cell line by means of the MTT-assay. Ethanol leaf extract have highest anticancer activity with an IC_{50} value of 141.56µg/ml. The results obtained from the analysis showed that the leaves of *A. muricata* has the property to inhibit the cell growth and it has medicinal properties and could be good source of drugs for treating cancer.

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INTRODUCTION

MEDICINAL PLANTS

A. muricata commonly known as Graviola or soursop belongs to the family of Annonaceae of the order Magnoliales and division Magnoliophyta. It is the most tropical semideciduous tree with the largest fruits of the Annona genus. It is widely distributed and native to sub-Saharan Africa countries. Over 70 species are classified under the genus Annona. Species A. muricata is found to be the most extensively grown. The Soursop tree grows up to 5–10 m tall and 15–83 cm in breadth with low branches. Ethnobotanical studies have indicated that A. muricata has been used as insecticide and parasiticide. Fruit juice and infusions of leaves or branches have been used to treat fever, sedative, respiratory illness, gastrointestinal problems, liver, heart, and kidney affections. In recent years it has become widely used for hypoglycemic, hypotensive and cancer treatments (Tisott et al., 2013).

MATERIALS AND METHODS

The present study mainly deals with the analysis of phytochemical constituents, antioxidant activity, antibacterial and anticancer activity found in leaves of *Annona muricata* and broadly evaluate its biochemical constituents, element composition and some secondary metabolites using standard protocols as follows.

COLLECTION OF SAMPLE *Annona muricata:* The Annona muricata (Graviola) leaves were collected from the local area in Erode district.

POWDERING OF THE SAMPLE *Annona muricata:* The collected Annona muricata leaves were subjected to shadow dried and it was further crushed to powder and the powder was stored in airtight container for further analysis.

EXTRACTION OF *Annona muricata:* The dried powdered plant material was extracted using Soxhlet assembly successively with Ethanol, Acetone and Aqueous. Each time before extracting with next solvent, the powdered material was dried in hot-air oven below 50ºC. The extracted materials were concentrated by evaporation.

ETHANOL EXTRACT OF *Annona muricata:* About 50gm of dry powder was extracted first with 250ml of ethanol at 60ºC to 80ºC by continues hot percolation using Soxhlet apparatus. After completion of extraction, ethanol was filtered and concentrated to dry mass by vacuum distillation. The extract was then stored in a desiccator.

PRELIMINARY PHYTOCHEMICAL SCREENING

Ethanol extract, Acetone extract, Aqueous extract of Annona muricata were subjected to qualitative tests for the identification of various active constituents like carbohydrates, alkaloids,anthraquinones, flavonoids, phenols, steroids, amino acids and protein.

TEST FOR CARBOHYDRATE

Molisch's Test

To few drops of plant extract, Molisch's reagent was added and concentrated sulphuric acid was added along the side of the test tube.

- Fehling Test

To a few drops of plant extract, Fehling's reagent was added and heated in a boiling water bath for few minutes.

- Benedicts Test

To few drops of plant extract, Benedict reagent was added and heated in the boiling water bath for few minutes.

- Picric acid Test

To few drops of plant extract, Picric acid solution was added and then few drops of 10% sodium carbonate was added and heated.

- Barford's Test

To few drops of plant extract, Barford's reagent was added and heated in boiling

TEST FOR ALKALOIDS

Draggendroff's Test

To few drops of plant extract, Draggendroff's reagent was added.

- Wagner's Test

To 2.3ml of test extract, few drops of Wagner's reagent was added.

- Hager Test

To few drops of plant extract, few drops of Hager reagent (1% picric acid) was added.

TEST FOR ANTHROQUINONE(a)BORNTARGGER'S TEST

To 2ml of extract, lml of Fecl2 and conc. Hcl was added, the solution was boiled and cooled, then the solution is filtered and mixed with few drops of Diethyl ether.**TEST FOR FLAVANOIDS**

LEAD ACETATE TEST

Few drops of extract were mixed with lml of 5% lead acetate solution.

SHINODA TEST

To 2.3ml of extract few fragments of magnesium metal was added in a test tube followed by dropwise addition of conc. Hcl/ TEST FOR PHENOLS (a)PHOSPHOMOLYBDIC ACID TEST. Few drops of extract were spotted on the filter paper and a drop of 5% phosphomolybdic acid was sprayed on it and then it was exposed to ammonia gas.TEST FOR STEROIDS

HALOGENATION TEST

In a test tube, add a drop of plant extract and few drops of bromine water.

SALKOWSI REACTION

To drop of extract, few drops of chloroform was added and then concentrated sulphuric acid was added along the sides of the test tube.

LIBERMANN-BURCHARD TEST

To 2ml of extract, added chloroform and 2ml of acetic anhydride and few drops of sulphuric acid mixed well and allowed it to stand for few minutes in dark.

TEST FOR PROTEIN

XANTHOPROTEIN TEST

The plant extract is treated with few drops of conc. HNO3

BIURETE TEST

To few drops of extract, few drops of Biuret reagent was added.

TEST FOR AMINOACIDS

NINHYDRIN TEST

Few drops of extract 1ml of 0.25% Ninhydrin reagent was added and kept inboiling water bath.

ELRISCH'S TEST

2ml of plantextract was added to the Elrisch reagentand kept in boiling water bath.

FOLIN'S PHENOL TEST

To 1ml of plant extract add 1ml of Folin's phenol reagent and add saturated sodiumcarbonate solution.

FERRIC CHLORIDE TEST

1ml of plant extract was added with 1ml of Ferric chloride and few drops of 0.5% copper sulphatesolution.

PHYSICAL CONSTITUENTS OF *Annona muricata*

ESTIMATION OF ASH CONTENT

About 1.0gm of sample was taken in two different crucibles. The crucibles were heated over Bursenburner until the sample turned into ash. Heating, cooling and weighing were repeated until constant weight was got.

ESTIMATION OF SECONDARY METABOLITES

- Estimation of alkaloid (Sigh and Archana sahu method)
- Estimation of flavonoid (Aluminium chloride method)
- Estimation of phenol (Mallick and singh., 1980)
- Estimation of tannin (Folin's phenol Method)

BIOCHEMICAL ANALYSIS

ESTIMATION OF CHLOROPHYLL (Arnon, 1956)

Chlorophyll is extracted with 80% acetone and the absorption is measured at 663 nm and645 nm in a spectrophotometer. Using the absorption coefficients, the amount of total chlorophyll present in the sample is calculated

- Estimation of glucose (Anthrone method)
- Estimation of protein (Lowry's method)
- Estimation of cholesterol (Zak's method)
- Estimation of free amino acids (Moore and Stein, 1948)

ESTIMATION OF VITAMINS AND MINERALS

- Estimation of vitamin c (Omaye et al., 1971)
- Estimation of iron by (2,2'-dipyridyl method)
- Estimation of magnesium (Titan Yellow Method)

EVALUATION OF SCAVENGING ACTIVITY OF *Annona muricata*

- DPPH' radical scavengingassay (Brand williams et al., 1995)
- Ferric reducing antioxidant power (Oyaizu, 1986)

STATISTICAL ANALYSIS

Three extracts of Annona muricata were analysed to determine their components. All experiments were done in triplicate, and mean values are presented. Results were expressed as Mean±SD (Standard Deviation).

ANTI-BACTERIAL ANALYSIS CULTURING OF BACTERIAL SAMPLES

1ml of bacterial samples Escherichia coli, Klebsiella, Streptococcus aureus and Bacillus subtilis are mixed with 5% peptone water. At 20ºC, 20ml of culture media is poured into the Petri plates containing the suspension. After the agar is set, under the aseptic condition, moistened a swab with 5% peptone water samples and spread it uniformly over the surface of the agar plate by using L-Rod.Incubate the petri dishes at 37ºC for 24 hours and kept the petri dishes with mother plate up. The grown cultures were stored at 37ºC for further studies and directly used.

ANTI-BACTERIAL ASSAY (Bauer et al., 1966): Microorganisms are naturally susceptible to the action of a specific antibiotic and are inhibited by it. The susceptibility is based on genetically determined characteristic of individual species. Disc methodis the simplest method to perform the susceptibility test. Discs of filter paper impregnated with antibiotic ate placed on an agar plate (i.e.) heavily and uniformly inoculated with an actively growing culture of the organism. The test organisms grow in a smooth 'lawn' of confluent growth on the plate except in a clear zone around the antibiotic disc, which inhibits the growth of the organism and indicates the susceptibility of the organism. At 20ºC, 20ml of culture media is poured into the petri plates containing the suspension. After the agar is set, under the aseptic condition, moistened a swab with 5% peptone water samples and spread it uniformly over the surface of the agar plate by using L-Rod. Removed each one of the antibiotic discs (Streptomycin, Kanamycin and Penicillin) from the container using the forceps and placed it gently on the surface of the incubated plate. Tapped the disc gently with the forceps to fix it in position and extracted plant fruit disc was placed surface of the agar plate. Incubate the petri dishes at 37ºC for 24 hours and kept the petridishes with right side up. After the incubation period, the zone of inhibition was determined. The result was obtained by measuring the zone of inhibition in mm/diameter.

IN VITRO CYTOTOXIC ACTIVITY BY MTT ASSAY

Cell line: The breast cancer cell line (MCF 7) was obtained from National Centre for Cell Science (NCCS),Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 370C, 5% CO2, 95% air and 100% relative humidity, Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure: The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x105 cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 370C, 5% CO2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethyl sulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resultingin the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 370C, 5% CO2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay: 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water-soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15 µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at370C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm usingmicro plate reader.

Calculation

(At − Ab) ÷ Cell survival = × 100(Ac − Ab)Where, At = Absorbance of Test
Ab= Absorbance of Blank (Media)Ac= Absorbance of control (cell)
% cell inhibition = 100 − % cell survival
Cell viability %: =Mean OD of wells receiving each plantextract dilution / Mean OD of control wells × 100.

RESULTS AND DISCUSSION PHYTOCHEMICAL SCREENING: Phytochemical screening of Annona muricata

The results of the preliminary phytochemical screening were shown in Table-1. Table-1 and Figure-1 shows the phytochemical analysis of various extracts of *Anona muricata* leaves All theextracts of *Annona muricata* exhibit the presence of Carbohydrate, Reducing sugar, Alkaloid, Protein, and steroids.

Physical constituents of *Annona muricata*

The physical characteristics of all the extracts of *Annona muricata* were shown in the Table-2 andFigure- 2. The physicalcharacteristics of *Annona muricata* leaves were shown in the Table-2 and Figure-2. The ash content of *Annona muricata* was 36% while the moisture content of *Annona muricata* was 59.6%. The moisture content is essential for the growth of plants.

Table 1. Qualitative phytochemical analysis of Anona muricata leaves

S.NO	EXPERIMENTS	ETHANOL	ACETONE	AQUEOUS
	TEST FOR CARBOHYDRATES			
1.	Molisch's Test	+	+	+
2.	Fehling's Test	+	+	+
3.	Benedict's Test	+	+	+
4.	Picric acid Test	+	+	+
5.	Barford Test	+	+	+
	TEST FOR ALKALOIDS			
6.	Draggendroff's's Test	+	+	+
7.	Hagner's Test	+	+	+
8.	Wagner's Test	+	-	+
	TEST FOR ANTHRAQUINONES			
9.	Bontrager's Test	-	-	-
	TEST FOR FLAVONOIDS			
10.	Shinoda Test	_	+	+
11.	Lead Acetate Test	_	_	+
	TEST FOR PHENOLS			
12.	Phosphomolybdic Acid Test	+	-	+
	TEST FOR STEROIDS			
13.	Libermann Burchard Test	+	+	+
14.	Halogenation Test	-	-	_
15.	Salkowski Test	-	_	_
	TEST FOR PROTEIN			
16.	Xanthoprotein Test	+	+	+
17.	Biurete Test	+	+	+
	TEST FOR AMINOACIDS			
18.	Ninhydrin Test	-	+	-
19.	Elrich's Test	_	+	_
20.	Folin's Phenol Test	_	+	_
21.	Ferric chloride		+	

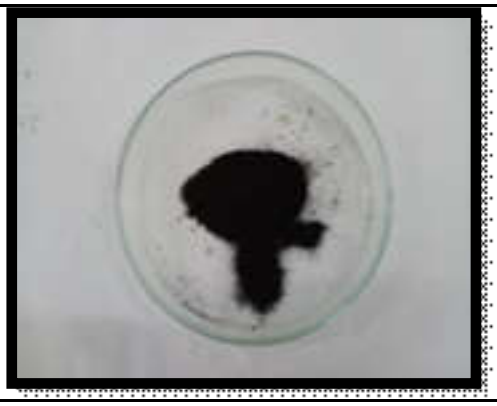


a b C

Figure 1: A) Aqueous ExtractB) Ethanol ExtractC) Acetone Extract

Table 2. Physical constituents of *Annona muricata* leaves

SAMPLE	ASH %	MOISTURE %
Annona muricata leaves	36	59.6




ASH **MOISTURE**

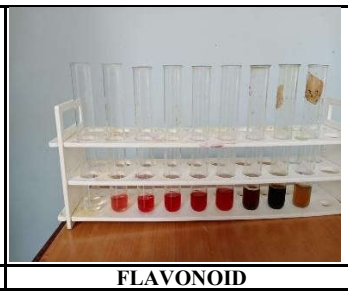
ESTIMATION OF SECONDARY METABOLITES

Estimation of alkaloids, flavonoids, phenols, and tannin in all the extract of *Annona muricata*

The amount of alkaloids, flavonoids, phenols and tannin in all the extracts of *Annona muricata* were estimated by colorimetric method. The results were shown in Table-3 and Figure-3.

Table 3. Estimation of alkaloids, flavonoids, phenol, and tannin in all the extract of *Annona muricata*

EXTRACTS	ALKALOIDS (mg/g)	FLAVONOIDS (mg/g)	PHENOLS (mg/g)	TANNIN (mg/g)
Ethanol	1.26±0.23	0.37±0.55	0.17±0.04	2.64±0.62
Acetone	1.25±0.24	0.35±0.57	1.16±0.04	2.60±0.67
Aqueous	1.24±0.24	0.33±0.53	1.15±0.06	2.41±0.91

ALKALOID **FLAVONOID** **PHENOL** **TANNIN**

Table-3 and Figure-3 of secondary metabolite estimation, shows that the ethanol extract of *Annona muricata* have higher secondary metabolites, Acetone has moderate concentration and aqueous has lower concentration. The presence of various secondary metabolites increases the medicinal values of the plant.

BIOCHEMICAL STUDIES ESTIMATION OF CHLOROPHYLL

The amount of chlorophyll was estimated by colorimetric method. The results were shown in Table-4 and Figure-4. Table 4 and Figure 4 of this study shows that the chlorophyll A was 0.03mg/g, chlorophyll B was 0.05mg/gand Total chlorophyll was 0.08mg/g. From the above analysis shows that the sample B has more chlorophyll content than sample A. Chlorophyll is the essential element for the photosynthetic process. Estimation of glucose, protein, cholesterol, and amino acids in various extract of *Annonamuricata* leaves The amount of Glucose, Protein, Cholesterol, Amino acid in various extract of *Annona muricata* leaves were estimated by colorimetric method.

Table 4: Estimation of chlorophyll in *Annona muricata* leaves

SAMPLE	CHLOROPHYLL (mg/g)		
	A	B	Total chlorophyll
Annona muricata	0.03±0.001	0.05±0.07	0.08±0.06

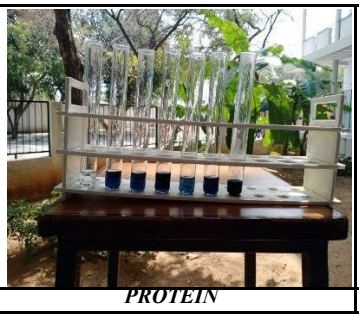
CHLOROPHYLL

The results were shown in Table-5 and Figure-5. Table 5 and Figure 5 shows the biochemical study of *Annona muricata* and this reveals that the higher biochemical contents were present in ethanol extracts. The various biochemical constituents help to increase the metabolic process of the plant.

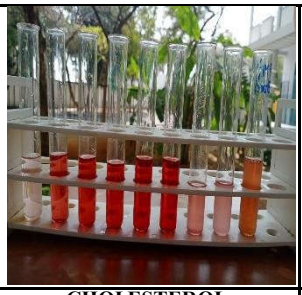
Table 5: Estimation of glucose, protein, cholesterol, and amino acid in various extract of *Annonamuricata*

EXTRACTS	GLUCOSE (mg/g)	PROTEINS (mg/g)	CHOLESTEROL (mg/g)	AMINO ACIDS (mg/g)
Ethanol	9.5±0.95	10.46±0.93	11.75±0.93	7.57±0.73
Acetone	8.53±0.78	8.33±0.90	11.17±0.39	7.40±0.81
Aqueous	8.30±0.81	6.39±0.96	10.50±0.62	5.58±0.90

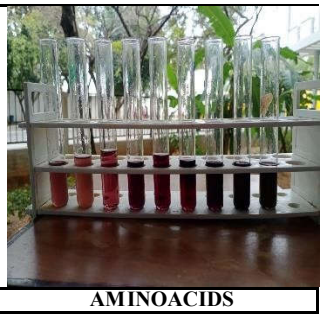
GLUCOSE



PROTEIN



CHOLESTEROL



AMINOACIDS

ESTIMATION OF MINERALS AND VITAMIN

Estimation of minerals and vitamin in various extract of *Annona muricata* leaves

The amount of Iron, Magnesium, and vitamin C in various extract of *Annona muricata* were estimated by colorimetric method. The results were shown in Table- 6 and Figure-6. Table 6 and Figure 6 shows the minerals and vitamin estimation in various extracts of *Annona muricata* which reveals that the ethanol extract has higher vitamin and mineral concentration. Different minerals play a major role in the growth and development of the plant.

Table 6. Estimation of minerals and vitamin in various extract of *Annona muricata* leaves

EXTRACTS	MINERALS (mg/g)		VITAMIN (mg/g)
	IRON	MAGNESIUM	VITAMIN C
Ethanol	9.29±0.55	4.43±0.70	12.47±0.70
Acetone	8.43±0.49	3.83±0.56	10.90±0.56
Aqueous	7.28±0.71	2.71±0.95	8.39±0.95

IRON

MAGNESIUM

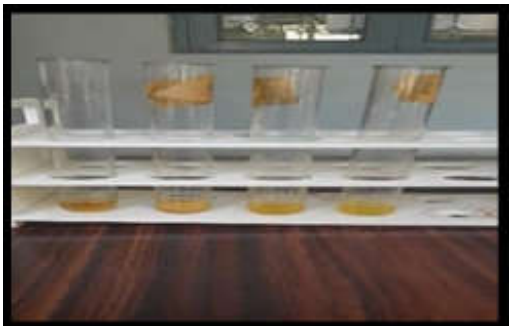
VITAMIN C

EVALUATION OF SCAVENGING ACTIVITY OF VARIOUS EXTRACT OF *Annona muricata* LEAVES. DPPH' radical scavengingassay

The free radical scavenging activity of various extract of *Annona muricata* was determined by DPPH method and result were presented in table-7 and figure-7. Table 7 and Figure 9 reveals the DPPH activity of *Annona muricata*, which shows that the Ethanol extract has higher scavenging activity. The free radical scavenging activity is important to prevent various disease.

Table 7. DPPH' radical scavenging assay in various extract of *Annona muricata*

EXTRACT	DPPH (%)
Ethanol	1.02±0.03
Acetone	0.96±0.09
Aqueous	0.72±0.16



FERRIC REDUCING ANTIOXIDANT POWER: FRAP activity in various extract of *Annona muricata* results were presented in table-8 and figure -8. Table 8 and Figure 8 reveals the FRAP activity of *Annona muricata*, which shows that the Ethanol extract has higher scavenging activity.

Table 8. Ferric reducing antioxidant power

EXTRACT	**FRAP (%)**
Ethanol	1.39±0.50
Acetone	1.37±0.50
Aqueous	1.38±0.50

ANTI-BACTERIAL ASSAY BY DISC DIFFUSION METHOD: The antibacterial spectrum of the ethanol extract of *Annona muricata* against Gram positive and Gram-negative bacterial samples was performed using disc diffusion method and the results were measured in mm. The results obtained were compared with the standard antibiotic discs such that are commercially available. The results were shown in the Table-9 and Figure-9. In the ethanolic extract, the Zone of inhibition against E. coli was 7mm, for Klebsiella pneumoniaewas 6mm, for S. aureus was 5mm and for B. subtilis was 4mm respectively. The antibacterial spectrum wasmore for E. coli.

Table 9. Zone of inhibition with ethanol extract of *Annona muricata*

Extracts	Zone of inhibition(mm)			
	Escherichia coli	Klebsiella pneumoniae	Streptococcus aureus	Bacillussubtilis
Ethanol	7.0±0.5	6.0±0.4	5.0±0.3	4.0±0.2

(A) Streptococcus aureus (B) Escherichia coli (C) Klebsiella pneumoniae (D)Bacillus subtilis

IN VITRO CYTOTOXIC ACTIVITY BY MTT ASSAY

In vitro cytotoxic studies were conducted for the ethanol extracts and results are showed in Table 10 and Figure 10. Table 10 and Figure 10 results showed decreased cell viability and cell growth inhibition in a dosedependent manner.

Table 10. In vitro cytotoxic activity of ethanol extract of *Annona muricata* on Breast cancer celllines (MCF 7 cell line)

Sample Conc(µg)	%Cell inhibition
18.75	11.04
37.5	25.78
75	36.83
150	51.56
300	62.61
IC50(µg/ml)	141.56

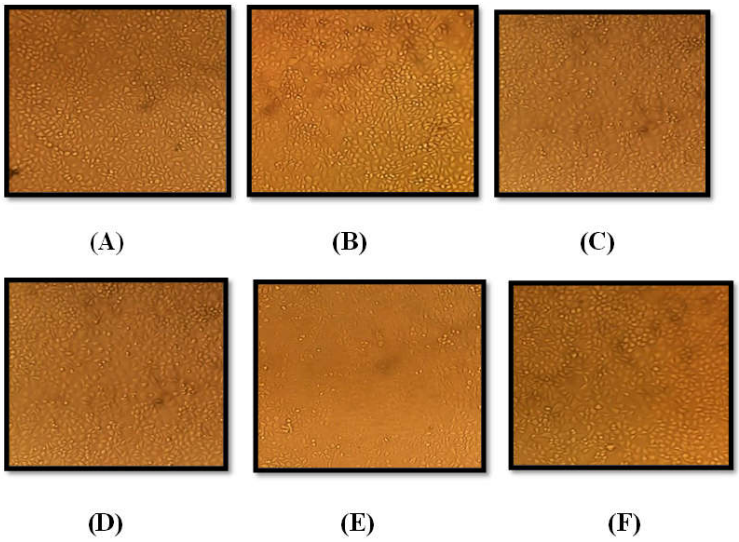


Figure 10. A)18.75µg B)37.5µg C)150µg D)75µg E)300µg F)MCF 7

SUMMARY AND CONCLUSION

Annona muricata is a tropical plant, that has been used in the ethnomedicine to treat wide range of diseases, from common cold to cancer. The qualitative screening of *A. muricata* leaf extracts revealed the presence of various bioactive components such as carbohydrate, reducing sugar, alkaloid, protein, and steroids. The phytochemical test showed that all the extract contained secondary metabolites compound with active constituents. The extracts were found to be rich in alkaloids which have wide pharmacological effects and thus have been used extensively as drugs in medical field. The extract revealed significant scavenging of FRAP and DPPH free radical in dose-dependentmanner and this may attribute to their electron donating ability. Then these observations show that *A. muricata* extracts might prevent reactive radical species from damaging biomolecules in susceptible biological and food system. *Annona muricata* has an antibacterial activity toward various pathogenic microorganisms which support its ethnomedicinal for the treatment of many infectious disease The current study showed that the ethanolic extract of *A. muricata* has important bioactive compounds which can be used in the management and treatment of cancer. The ethanolic extract of *Annona muricata* leaves displayed strongest cytotoxic properties against the MCF-7 breast cancer cell line. In conclusion, our results suggest that the various increasing concentration of ethanol extracts of *Annona muricata* leaves significantly reduced cell proliferation in MCF-7 breast cancer cell line. My future study is focused on using the fruits and seeds of *A. muricata* for treating breast cancer cell line.

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