A Report Of
In Vitro Anti-cancer activity of Ocimum Sanctum Callus Extract

:: Submitted To::

Satej Global Science

Suvas Apartment,

Behind Park Avenue Bunglow, Thaltej,

Ahmedabad-380059, Gujarat, India.

:: Submitted By ::

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Report of "*In Vitro* Anti-cancer activity of *Ocimum Sanctum* Callus Extract"

Name of Product	*Ocimum Sanctum* Callus Extract
Method Followed	Cytotoxicity study by MTT Assay (ISO 10993-5:2009)
Testing Facility	Accuprec Research Labs Pvt. Ltd. Opp. Zydus Pharmez, Changodar- Bavla Highway, Nr. Matoda Patiya, Post- Matoda, Ahmedabad, Gujarat 382213, India.
Sponsor	Satej Global Science Suvas Apartment, Behind Park Avenue Bunglow, Thaltej, Ahmedabad-380059, Gujarat, India.
Study Period	4 Days
Turn around period	14 Days
Report Number	ARL/3994/2017
Report Date	04/11/2017

Prepared By (Sign/Date)	Reviewed By (Sign/Date)	Approved By (Sign/Date)
Ms. Kruti Upadhyay (Research Assistant)	Dr. Manish Rachchh (CEO)	Dr. Rina Gokani (CSO)
04/11/2017	04/11/2017	04/11/2017

TABLE OF CONTENTS

I. STATEMENT OF COMPLIANCE

We, the undersigned hereby declare that the study entitled "*In Vitro* Anti-cancer activity of *Ocimum Sanctum* Callus Extract" was performed under our supervision in compliance with OECD principles of Good Laboratory Practices (OECD, 1998) & ISO17025. Characterization of the test material was performed by the sponsor. The objective laid down in the study protocol was achieved. No unforeseen circumstances were observed which might affect the quality or integrity of the study.

The report represents a true and accurate results obtained. We accept the responsibility for validity of the data, as well as the interpretation, analysis, documentation and reporting of the results.

The report comprises of total 16 pages and includes statement of compliance, quality assurance statement, study personnel detail, experimental design, results, discussion, conclusion, reference and period of archival.

Date: 04/11/2017

04/11/2017
Ms. Kruti Upadhyay
Asst. Study Director

04/11/2017
Dr. Manish Rachchh
Study Director

04/11/2017
Mr. Bhargav Gohel
DM, QA

04/11/2017
Dr. Rina Gokani
Q. A. Head

II. QUALITY ASSURANCE STATEMENT

This study report has been reviewed by the Quality Assurance Unit of Accuprec Research Labs Pvt. Ltd., for compliance with the OECD Principles of GLP & ISO 17025.

This statement confirms that the study report accurately reflects Study data. The summary of inspections performed during the course of the study is as follows:

Sr. No.	Type of Inspection	Date of Inspection	Phases of Study inspected
1	Study Based	01/11/2017	Preparation of test sample
2	Study Based	02/11/2017	Evaluation of cell Viability

Date: 04/11/2017

04/11/2017

Mr. Bhargav Gohel
DM, QA

04/11/2017

Dr. Rina Gokani
Q. A. Head

III. ABBREVIATION

GLP - Good Laboratory Practice
Gm - Gram
hr - Hour
ISO - International Organization for Standardization
Kg - Kilogram
Mg - Milligram
MSDS - Material Safety Data Sheet
EDTA - Ethylenediaminetetraacetic acid
IPA - Isopropyl Alcohol
FBS - Fetal Bovine Serum
MEM - Minimum Essential Medium
NCCS - National Centre for Cell Science

1. STUDY INFORMATION

Report No. : ARL/3994/2017

Study Title :"*In Vitro* Anti-cancer activity of *Ocimum Sanctum* Callus Extract".

Sponsor : Satej Global Science
Suvas Apartment,
Behind Park Avenue Bunglow, Thaltej,
Ahmedabad-380059, Gujarat, India.

Testing Facility : Accuprec Research Labs Pvt. Ltd.
Opp.Zydus Pharmez,
Changodar- Bavla Highway,
Nr. Matoda Patiya, Post- Matoda,
Ahmedabad, Gujarat 382213, India

Name of Asst. Study Director : Ms. Kruti Upadhyay
Sign of Asst. Study Director : 04/11/2017

Name of Study Director : Dr. Manish A. Rachchh
Sign of Study Director : 04/11/2017

Name of DM - Q. A. : Mr. Bhargav Gohel
Sign of DM - Q. A. : 04/11/2017

Name of Q. A. Head : Dr. Rina Gokani
Sign of Q. A. Head : 04/11/2017

2. SUMMARY

The objective of the study now reported was to determine *in vitro* anti-cancer activity of "*Ocimum Sanctum* Callus Extract", by performing cytotoxicity test by MTT assay using Caco-2 cell line.

3. INTRODUCTION

3.1. OBJECTIVE

The study was conducted to establish *in vitro* anti-cancer activity of the *Ocimum Sanctum* Callus Extract using Caco-2 cell line.

3.2. TEST GUIDELINES:

Cytotoxicity test by MTT assay using Caco-2 cell line.

4. STUDY PERSONNEL

Study Director	**: Dr. Manish A. Rachchh**
Research Assistant	**: Ms. Kruti Upadhyay**
Research Assistant	**: Ms. Kanchan Khare**
Research Assistant	**: Mr. Amol Kharat**
DM - Q.A.	**: Mr. Bhargav Gohel**
Q. A. Head	**: Dr. Rina Gokani**

5. MATERIALS AND METHODS

5.1. TEST ARTICLE DETAILS

Test Article	*Ocimum Sanctum* Callus Extract
Storage Conditions	The test article was stored at room temperature.
Stability	The stability of the test article formulations, under the storage conditions used in this study, is the responsibility of the Sponsor.
Safety Precautions	Standard laboratory safety procedure was employed for handling the dose formulations. Specifically, gloves and eye protection were worn while administering doses.
Date of Initiation of Study	28/10/2017
Date of Completion of Study	02/11/2017

5.2. MATERIALS

Sr. No.	Name of material	Type	Make
A	Caco-2, Human epithelial cells	Cell line	NCCS
B	Minimum essential medium	Medium	Hi Media
C	Phosphate Buffer pH 7.4	Medium	Hi Media
D	Fetal Bovin Serum	Medium	Hi Media
E	Trypsin -EDTA	Medium	Hi Media
F	Antibiotic (Streptomycin)	Reagent	Hi Media
G	Test Sample	-	Provided by Sponsor

5.3. Instruments:

1. CO_2 Incubator
2. Water bath
3. Inverted Microscope
4. Biosafety Cabinet
5. Microplate Reader

5.4. Preparations:

Test:

The test sample was prepared with phosphate buffered saline at different concentrations such as 10µL/mL, 50µL/mL, 100µL/mL, 500µL/mL and 1000µL/mL (undiluted extract); immediately prior to use.

Positive Control:

Cyclophosphamide (2 mg/ml) was freshly prepared in complete medium (MEM medium + 10% FBS) immediately prior to use. When necessary, pH was adjusted to 7.4 using 1 N HCl or 1 N NaOH.

Blank:

Complete medium (MEM medium + 10% FBS) having 2% antibiotic in incubator at 37°C in 5% CO_2 under aseptic condition for 24 hours.

Negative Control:

Phosphate buffered saline was used as negative control.

MTT Solution:

MTT was prepared in fresh PBS at a concentration of 1 mg/ml and adjust the pH 6.0-6.5. Solution is sterilized by sterile filtration using syringe filters (pore size 0.22 µm). The solution should be used on the same day.

6. EXPERIMEMNTAL DESIGN

6.1. Sub culturing of cell line

- **Detachment of cells**

Cell culture flask was removed from deep freezer and immediately thawed by keeping the flask in water bath at 37°C. The process of thawing was performed fast so that during thawing the cells did not get damaged. After the culture was thawed , the flask was removed from water bath and sprayed with 70% IPA on outer surface so that the outer exposed surface got disinfected and then the flask was placed in laminar air flow. 3-5 ml of 0.25% trypsin-EDTA was added in T-25 flask to detach cells from the flask under sterile condition in aseptic cabinet. The flask was shaken in horizontal direction so that each and every cell got exposed to trypsin solution. Flask was then incubated for 2-3 min at 37°C, 5% CO_2. The flask was observed under microscope to see whether cells were detached. Detached cells appeared in round shape and floated in the medium. After incubation immediately 20% FBS was added to stop the detachment process.

- **Viable cell count**

Counting of cells per ml of medium was carried out using hemocytometer. 10µl of cell suspension was taken and 20µl of trypan blue dye (0.5%) was added. Mixture of cell suspension and dye was dropped on hemocytometer. The unstained (viable) and stained (non viable) cells were counted separately, % cell viability was calculated.

6.2. Splitting of culture cells

After cell calculation the cells were splitted to half in two different sterile centrifuge tubes. In each tube the volume of fresh medium was added in such a way that the final concentration of cells in the medium would be (approximately: 4.25 X 10^5) in T-25 flask. The culture was transferred in T-25 flask and incubated for 4 Days in humidified atmosphere in incubator maintained at 37^0C temperature and 5% CO_2.

6.3. Preparation of Test extract

The test sample was prepared with phosphate buffered saline at different concentrations such as 10µL/mL, 50µL/mL, 100µL/mL, 500µL/mL and 1000µL/mL (undiluted extract); immediately prior to use.

Preparation of Blank, Positive Control and Negative Control:

Two T 25 flasks having 2 ml cell suspension, 2 ml fresh complete medium having 2 % antibiotic were prepared. Further, 2 ml fresh complete medium having 2 % antibiotic was added in control flask. The flasks were then incubated in incubator at 37°C in 5% CO_2 for 48 h.

6.4. Evaluation of Cytotoxicity:

i) Microscopic examination:

Following incubation, the culture wells were examined microscopically to evaluate cellular characteristics and % lysis.

Each Flask was evaluated for % lysis and cell characteristics and categorized as per below:

Grade	Reactivity	Condition of cell culture
0	None	No lysis
1	Slight	Occasional lysis
2	Mild	No extensive lysis
3	Moderate	Not more than 70 % of cell layers contain rounded cells or are lysed
4	Severe	Nearly complete cell lysis

ii) MTT assay

Step I: Growing cells in 96 well micro titer plate

Cell cultures was removed from culture flasks by enzymatic digestion (trypsin/EDTA). Dispense 100 µl culture medium only (blank) into the peripheral wells of a 96-well tissue culture microtitre plate. In the remaining wells, dispense 100 µl of a cell suspension of 1×10^5 cells/ml (= 1×10^4 cells/well). Incubate cells for 24 h (5% CO_2, 37°C) so that cells form a half-confluent monolayer. This incubation period ensures cell recovery, and adherence and progression to exponential growth phase. Each plate was examined under a phase contrast microscope to ensure that cell growth was relatively even across the microtitre plate.

STEP II: Contact of cells with test extract

After 24 h incubation, culture medium was aspirated from the cells. Per well, 100 µl of treatment medium containing either the appropriate concentration of sample as well as positive control, negative control and in three well (control) where 100 µl fresh medium was added. Cells were incubated for 24 h (5% CO_2, 37°C).

Step III: Examination of Cytotoxicity

After 24 h treatment, each plate was examined under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells.

After the examination of the plates, the culture medium from the plates was carefully removed. 50 µl of the MTT solution (1 mg/ml & pH 6.0-6.5) was then added to each test well and the plates were further incubated for 4 h in the incubator at 37°C. MTT solution was descanted and 100 µl of Dimethyl sulphoxide was added in each well. Well plate was transferred to a microplate reader equipped with a 570 nm filter to read the absorbance (reference wavelength 650 nm).

Step IV: Data analysis

A decrease in number of living cells results in a decrease in the metabolic activity in the sample. % cell Viability was calculated from following equation:

$$\% \text{ cell viability} = \frac{OD_{570e} \times 100}{OD_{570b}}$$

Where, OD_{570e} is the mean value of the measured optical density of the test sample as well as positive control;

OD_{570b} is the mean value of the measured optical density of negative control .

Interpretation:

In microscopic examination, achievement of numerical grade less than 2, based on Tables 1 of. 6.4, is considered a non-cytotoxic effect. The lower % cell viability value indicates the higher Cytotoxic potential of the test item. If viability is reduced to < 70 % of the negative control then it confirm that the test material possess Cytotoxic potential.

7. RESULTS

MTT ASSAY

The test sample was evaluated for its cytotoxicity against human epithelial cell line (Caco-2) using MTT assay. Different concentrations of the extract, such as; 10, 50, 100, 500 and 1000 µl/ml shows 58.50%, 66.75%, 65.49%, 82.74% and 84.63% cytotoxicity towards Caco-2 cell line, respectively.

Further, standard sample (Cyclophosphamide) showed 100 % cytotoxicity (**Annexure 1**).

8. DISCUSSION:

In the present study, in vitro cytotoxicity testing of *Ocimum Sanctum* Callus Extract was performed using human epithelial cell line (Caco-2). The test sample was subjected to cell proliferation assay by MTT. The results revealed that the extract showed significant cytotoxicity on Caco-2 cell line. Overall, the cell growth inhibition by the extract observed in this study was concentration dependant. Undiluted extract has shown 84.63% cytotoxic potential against Caco-2 cell line.

9. CONCLUSION:

It can be concluded that the test materials *Ocimum Sanctum* Callus Extract has shown cytotoxic potential under the prescribed test conditions. Maximum cytotoxicity observed was 84.63% by undiluted extract of *Ocimum sanctum.*

04/12/2017

Study Director

Dr. Manish A. Rachchh

10. REFERENCE

Roy M, Chakrabarty S, Siddiqi M, Bhattacharya RK. Induction of apoptosis in tumor cells by natural phenolic compounds. Asian Pacific J Cancer Prev2002;3:61–7.

11. LIST OF ATTACHMENT

Annexure	Content
Annexure 1	% cell viability (MTT Assay)
Annexure 2	Microscopic Examination
Annexure 3	Photographs of Caco-2 Cell Line (Blank, Positive Control, Negative Control and Test)

12. ARCHIVES

All original raw data, draft study plan and report, approved study plan and final report will be retained in the archives of Accuprec Research Labs Pvt Ltd, Ahmedabad, Gujarat, India for a period of 5 years. At the end of the archiving period, the Sponsor's instructions will be sought either to extend the archiving period or to return or dispose of the archived material.

Date: 04/11/2017

04/11/2017

Ms. Kruti Upadhyay
Asst. Study Director

04/11/2017

Dr. Manish Rachchh
Study Director

04/11/2017

Mr. Bhargav Gohel
DM, QA

04/11/2017

Dr. Rina Gokani
Q. A. Head

IV. CERTIFICATE

This is to certify that the test material "*Ocimum Sanctum* Callus Extract", supplied by Satej Global Science, Suvas Apartment, Behind Park Avenue Bunglow, Thaltej, Ahmedabad-380059, Gujarat, India., has been tested for *in vitro* anti-cancer activity using Caco-2 cell line.

The test material was found to be "Cytotoxic " on human epithelial cell line (Caco-2). This indicate that it possess anti-cancer property. Maximum cytotoxicity observed was 84.63% by undiluted callus extract.

Accuprec Research Labs Pvt. Ltd., is approved by the Food & Drug Administration, Gujarat State, Gandhinagar, through License No. GTL/37/31.

Date: 04/11/2017

04/11/2017

Ms. Kruti Upadhyay
Asst. Study Director

04/11/2017

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04/11/2017

Dr. Rina Gokani
Q. A. Head

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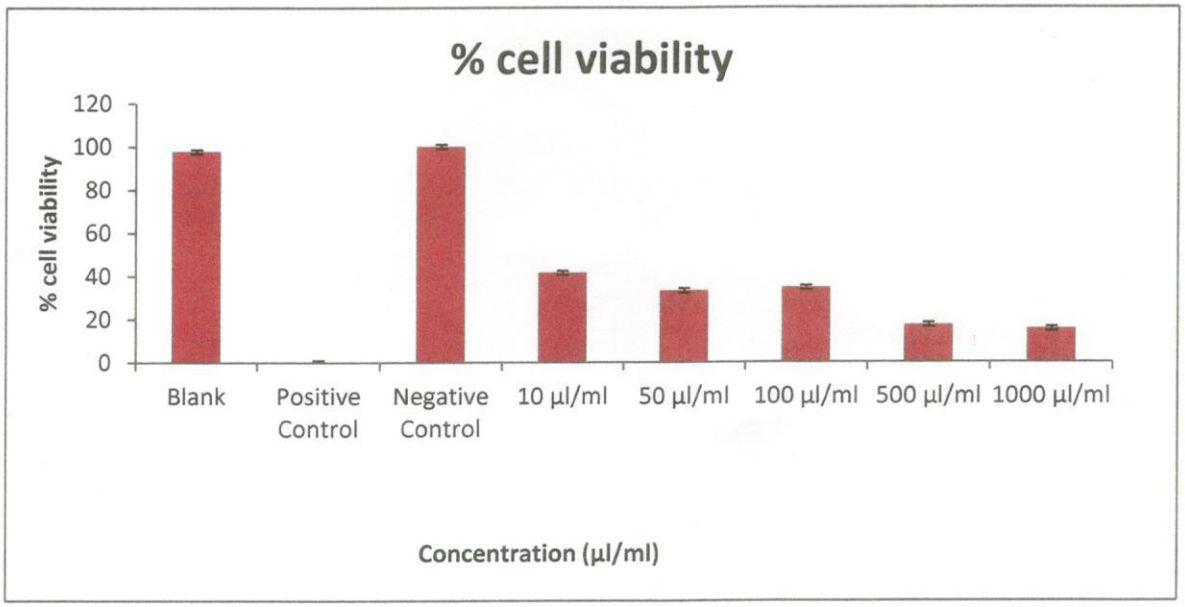
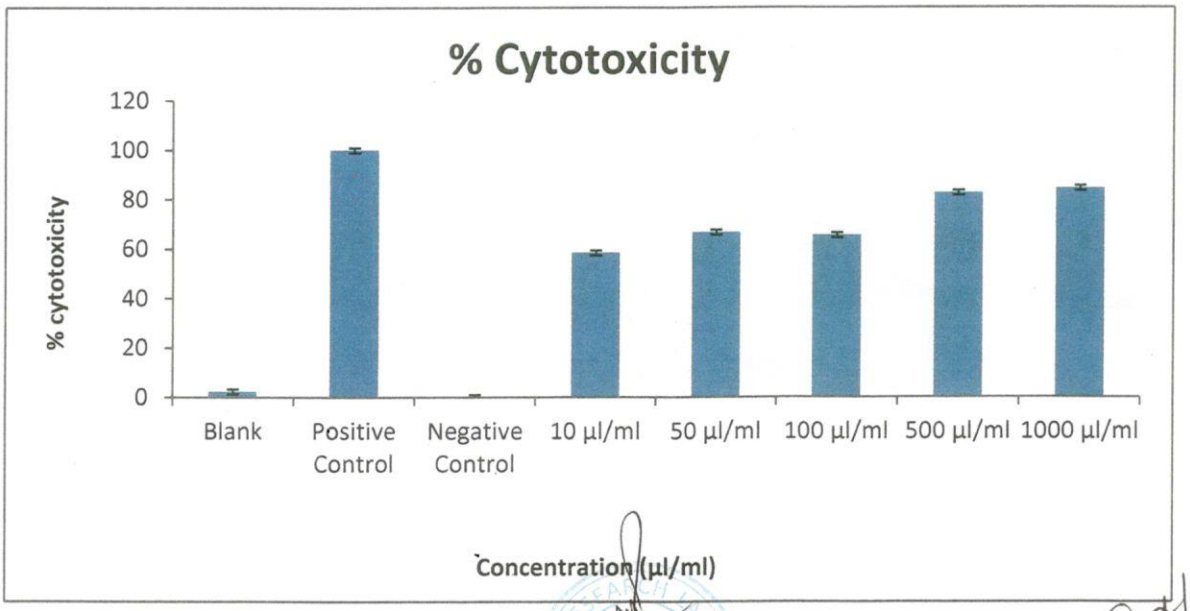
Annexure 1

Study Title: *In Vitro* Anti-cancer activity of Ocimum Sanctum Callus Extract.

Optical Density								
Sample	Blank	Positive Control	Negative Control	Test				
				10 µl/ml	50 µl/ml	100 µl/ml	500 µl/ml	1000 µl/ml (Undiluted Extract)
1	0.542	0.000	0.527	0.207	0.218	0.203	0.093	0.084
2	0.498	0.000	0.548	0.214	0.123	0.153	0.097	0.079
3	0.512	0.000	0.513	0.238	0.187	0.192	0.084	0.081
Avg.	0.517	0.000	0.529	0.220	0.176	0.183	0.091	0.081
% cell viability	97.733	0.000	100.000	41.499	33.249	34.509	17.254	15.365
% cell cytoxicity	2.267	100.000	0.000	58.501	66.751	65.491	82.746	84.635





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Annexure 2

Study Title: *In Vitro* Anti-cancer activity of Ocimum Sanctum Callus Extract.

Grade								
Sample	Blank	Positive Control	Negative Control	Test				
				10 µl/ml	50 µl/ml	100 µl/ml	500 µl/ml	1000 µl/ml (Undiluted Extract)
1	0.000	4.000	0.000	2.000	2.000	3.000	3.000	3.000
2	0.000	4.000	0.000	2.000	3.000	2.000	3.000	4.000
3	0.000	4.000	0.000	2.000	2.000	2.000	3.000	4.000
Avg.	2.000	2.000	1.000	2.167	2.333	2.667	3.333	3.667

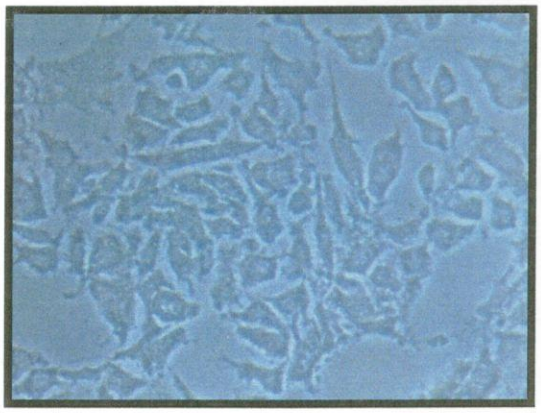
04/11/2017

Annexure 3

Microscopical examination of Anti-cancer activity of *Ocimum Sanctum* Callus Extract performed using Human epithelial cell line (Caco-2) following ISO 10993-5:2009 guideline.

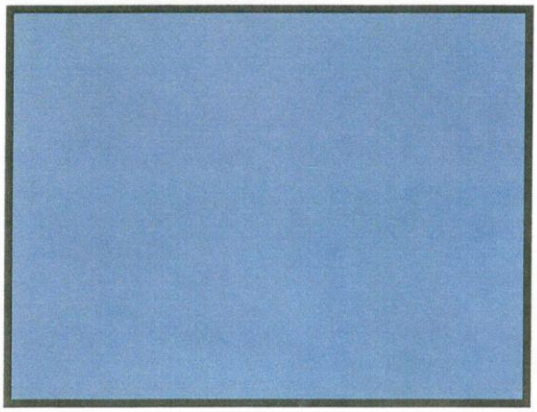
Sample Name: *Ocimum Sanctum* Callus Extract

Blank



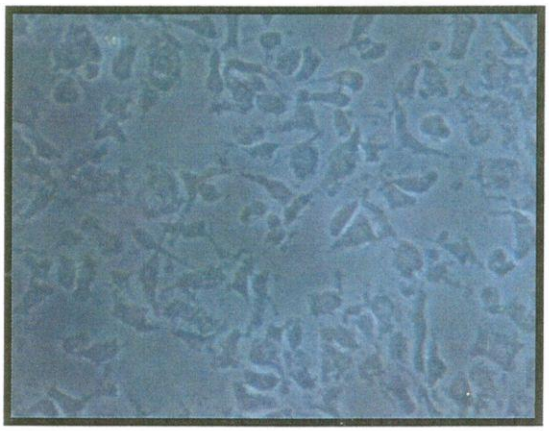
Morophology of control cells (magnification 20 X)

Positive Control (Cyclophosphamide)



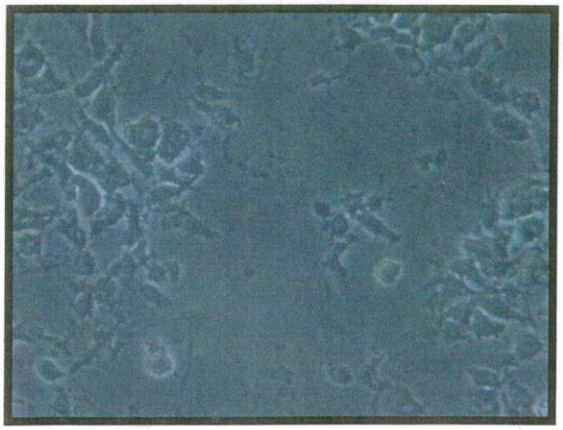
Morophology of Positive Control (magnification 20 X)

Negative Control (Phosphate Buffered Saline) Extract)



Morophology of Negative Control (magnification 20 X)

Test (10µL/mL) (*Ocimum Sanctum* Callus

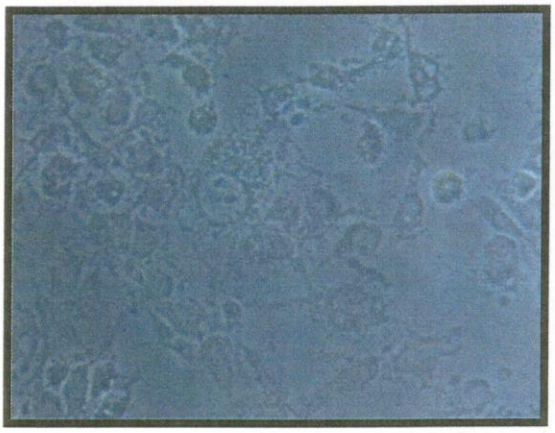


Morophology of Test (*Ocimum Sanctum* Callus Extract) treated cell (magnification 20 X)

04/11/2017

Test (50µL/mL)

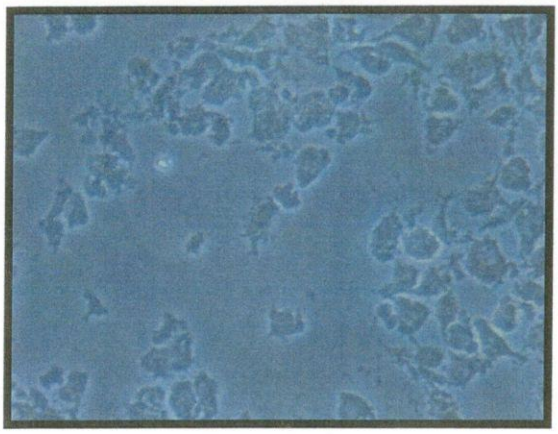
(Ocimum Sanctum Callus Extract)



Morophology of Test (*Ocimum Sanctum* Callus Extract) treated cell (magnification 20 X)

Test (100µL/mL)

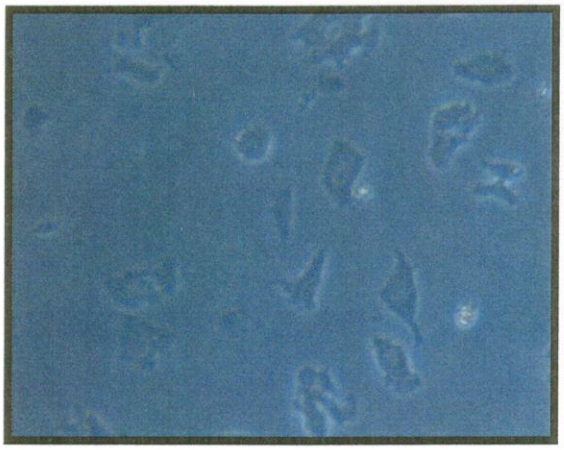
(Ocimum Sanctum Callus Extract)



Morophology of Test (*Ocimum Sanctum* Callus Extract) treated cell (magnification 20 X)

Test (500µL/mL)

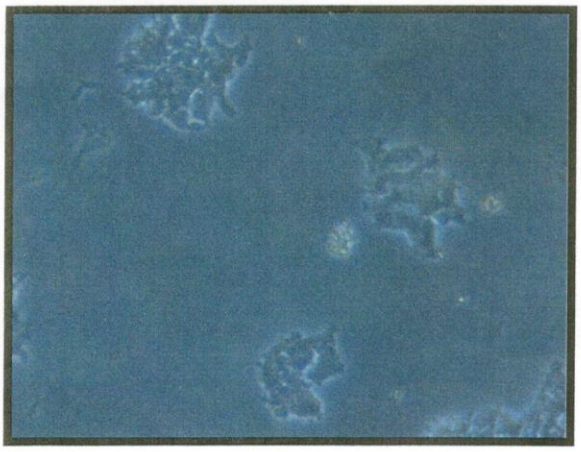
(Ocimum Sanctum Callus Extract)



Morophology of Test (*Ocimum Sanctum* Callus Extract) treated cell (magnification 20 X)

Test (1000µL/mL)

(Ocimum Sanctum Callus Extract)



Morophology of Test (*Ocimum Sanctum* Callus Extract) treated cell (magnification 20 X)

04/11/2017