

Pharmacognostical and phytochemical analysis of 10% aqueous extract of leaves of *shigru* (*Moringa oleifera* lam.), along with its *in vitro* comparative study in different cell lines of oral cancer

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Abstract

Most common and fast spreading disease in the present era is CANCER. Oral cancer ranks in the top three of all cancers in India, which account for over 30 % of all cancer reported in the country. Cancers are the most common cause of death in adults. Oral cancer is any malignant neoplasm which is found in oral cavity. The incidence of oral cancer is highest in India, South and Southeast Asian countries. In India, 90-95% of the oral cancers are squamous cell carcinoma. Cancer is really having a limited line of treatment and its prognosis; the fate is also unpredictable. It could be said that this is the disease of modernisation. But since it is not true as such incurable tumours like *Raktarbuda* and *Mansarbuda* have been described around 1500 B.C. by *Acharya Charaka* and *Ayurvedic* surgeon Acharya Sushruta.

Nowadays research is going to invent an *Ayurvedic* medicine to prevent or to cure cancer; *Shigru* (*Moringa oleifera* Lam.) is one of the most important drugs having anthraquinones, flavonoids, sitosterol, tannins, glycosides which possess the anticancer property. According to *Bhavprakash Shigru* is useful in the treatment of *Apachi*, *Gulma* and *Ganda*. *Raj Nighantu* mentioned *Shigru* as *Mukhjadyahar* (oral disorder). This study focuses on anticancer property of *Shigru* leaves on different cell lines of oral cancer by *in vitro* study.

Keywords: Shigru; *Moringa oleifera* Lam; In vitro study Shigru; Oral cancer; Aqueous extract

1 Introduction

Oral cancer is most common form found in India; in fact, it is among top three cancers. *Shigru* (*Moringa oleifera* Lam) belongs to family *Moringaceae* and is commonly known as ‘horse radish’ tree, ‘drumstick’ tree, ‘ben-oil tree’ or ‘benzoil tree’, ‘cabbage tree’, ‘mother’s best friend’ and ‘miracle tree’ in English. [6] A study conducted by Sreelatha s., Jeyachitra A., and Padma P.R. on leaves extract of *Shigru* has concluded that the leaves extract has a potential for cancer chemoprevention & can be claimed as a therapeutic target for cancer. [7] The *Shigru* (*Moringa oleifera* Lam) is widely spread and easily available in India, it is cheap and easy to use. Previous study has proved that *Shigru* possess anticancer property. Also, its chemical constituents point to its antitumor property. In vitro study was chosen for study because,

- Reduce costs,
- More directly assess product performance,
- Offer benefits in terms of ethical considerations.
- Adulteration in Ayurveda drugs is very common in today’s era.

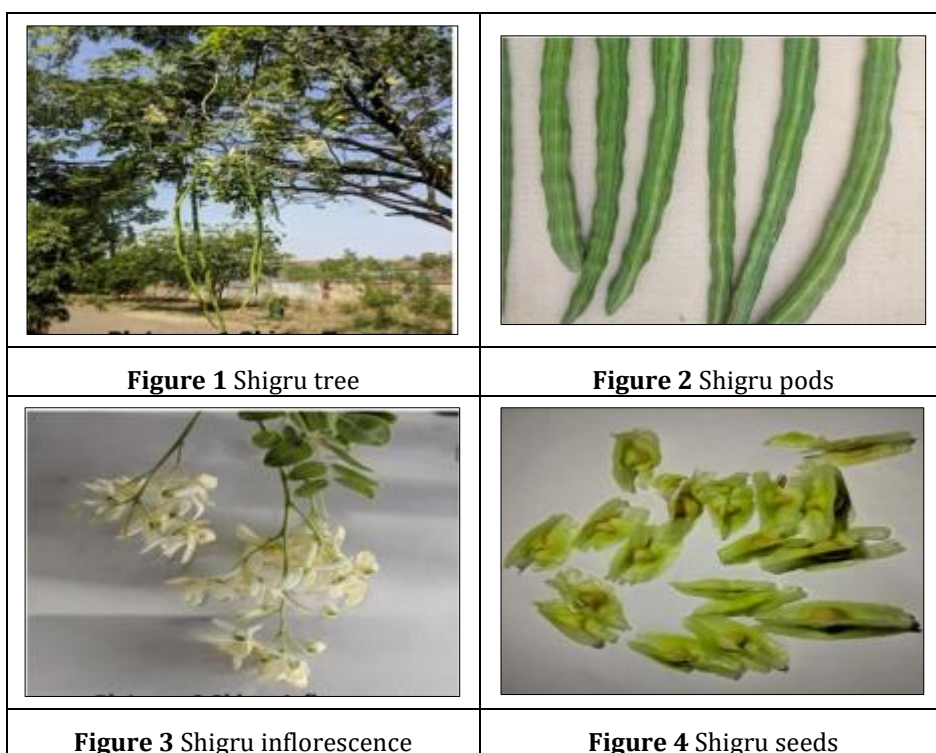
so, standardization of a drug is important step before conducting any study, so a complete standardization of *Shigru* (*Moringa oleifera* Lam) leaves had been done.

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2 Material and methods

For authentic data relating to various aspects of *Shigru*, an information is collected from Veda, Samhita, Nighantu, Kosha, pharmacopoeia, previous research works etc.

- Scientific classification
- Sanskrit Name: *Shigru*
- Botanical name – *Moringa oleifera* Lam
- Etymological Derivation of Botanical Name –
- Moringa: From the Malayalam Muringa or Tamil murungai –
- Oleifera: From the Latin oleum, olei-fero, meaning oil-bearing, in reference to the seeds of the species.
- Family – Moringaceae.



2.1 Pharmacognostical study

The present study is to explore the physical, chemical, biochemical, biological properties of drug *Shigru* (*Moringa oleifera* Lam) leaves.

3 Preparation of drug

The present study was carried out under following headings

3.1 Section I: Drug preparation and its standardization

It includes,

- Raw drug standardization,
- In process standardization,
- Finish product standardization

3.1.1 Part 1: raw drug standardization

Raw drug standardization was done in following steps

- Collection of sample.
- Macroscopy of *Shigru* (*Moringa oleifera* Lam) leaves.
- Organoleptic evaluation of *Shigru* (*Moringa oleifera* Lam) leaves.
- Microscopy of *Shigru* (*Moringa oleifera* Lam) leaves.
- Quantitative Microscopy of fresh plant leaves:
 - Determination of Stomatal Index
 - Preparation of powder drug
 - Physico-chemical analysis
 - Determination of foreign matter

Collection of Sample

The botanically identified sample of *Shigru* (*Moringa oleifera* Lam) leaves was collected from local area. And subjected for above raw drug standardization.

Macroscopy of *Shigru* (*Moringa oleifera* Lam) leaves:

Procedure: The external features of the test sample of *Shigru* (*Moringa oleifera* Lam) leaves were examined by naked eyes, using magnifying lens documented using Nikon 3300 digital camera. In the description, general morphology, habit, salient features of the plant and its useful parts are noted.

Organoleptic evaluation of *Shigru* (*Moringa oleifera* Lam) leaves

Procedure

The organoleptic evaluation of test sample of *Shigru* (*Moringa oleifera* Lam) leaves were done by *panchendriya pareeksha* of Ayurvedic classics. Organoleptic characters of the drug sample like color, odour, taste and texture, were observed and noted.

Microscopy of *Shigru* (*Moringa oleifera* Lam) leaves:

Procedure:

The fresh leaves of *Moringa oleifera* Lam. Were separated and washed thoroughly. Thin transverse sections of the leaves were taken using freehand sectioning technique, temporarily double stained with safranin and hematoxylin by the standard procedure. To find out the nature of cell wall, cell inclusion etc. The slides were observed under microscope with different magnifications, anatomical characteristics of the leaves were noted down and photomicrographic records were made.

Quantitative Microscopy of fresh plant leaves

Determination of Stomatal Index:

$$\text{Stomatal Index (I)} = \frac{S}{S + E} \times 100$$

Where, S: Number of stomata per unit area E: Number of ordinary epidermal cells in the same unit area.

3.1.2 Part 2: in process drug standardization

- Determination of moisture content value
- Determination of total ash
- Determination of acid insoluble ash
- Determination of pH value
- Determination of water-soluble extractive
- Determination of alcohol soluble extractive
- Determination of heavy metal: Arsenic, Cadmium, Copper, Lead, Mercury
- Physicochemical parameters

Table 1 Physicochemical parameters test (Raw material) of *Shigru*

Sr no	Tests	API Standard value	Powder of Shigru Leaves
1	Loss on drying	-	1.42%
2	Total Ash	Not more than 18 per cent	1.2177% w/w
3	Acid Insoluble Ash	Not more than 10 per cent	0.1098% w/w
4	Water Soluble Ash	-	0.78% w/w
5	Alcohol Soluble extractive	Not less than 3 per cent	8.4775% w/w
6	Water Soluble Extractive	Not less than 11 percent	13.4249% w/w
7	pH	-	4.25

3.1.3 Part: 3 final drug standardizations:

Preparation of extract ⁽⁸⁾

- **Selection of plant material**

The healthy, diseased free *Shigru* leaves were collected from local area; the leaves then spread in shade on white cloth in a well ventilated, insect, rodent and dust free lab room. The dried leaves were then ground into coarse powder.

- **Drying procedure**

Exposure to direct sunlight was avoided to prevent the loss of active components. The leaves were allowed to dry for 10 days.



Figure 5 Dry Leaves of Shigru

Preparation of coarse powder

The dried *Moringa oleifera* leaf powder was used to prepare the aqueous extract using the maceration method.

Preparation of aqueous extract ⁽⁹⁾

- One kilogram (1000 g) of dried *Moringa oleifera* leaf powder was accurately weighed on an analytical balance and poured into a 5000 ml pot.
- Four litres (4000 ml) of water were then gradually added to coarse powder with gentle shaking of the pot until slurry of uniform consistency was formed.
- The preparation was then kept on shaker for a period of 24 hrs for the purpose of frequent agitation until the soluble matter has dissolved.
- The mixture then is strained, (the damp solid material is pressed) using plastic filter mesh and using three layers of muslin cloth. This liquid extract is again filtered using double Whatman's filter paper.
- Finally, the liquid extract is poured into clean plates and allowed to dry in shade which is insect, rodent and dust free, well-ventilated room.

- This extract is kept in shade till the water get completely evaporated and a dark brown coloured somewhat sticky extract was formed.
- This concentrated dried extract was then collected into light resistant bottles and freeze-dried.

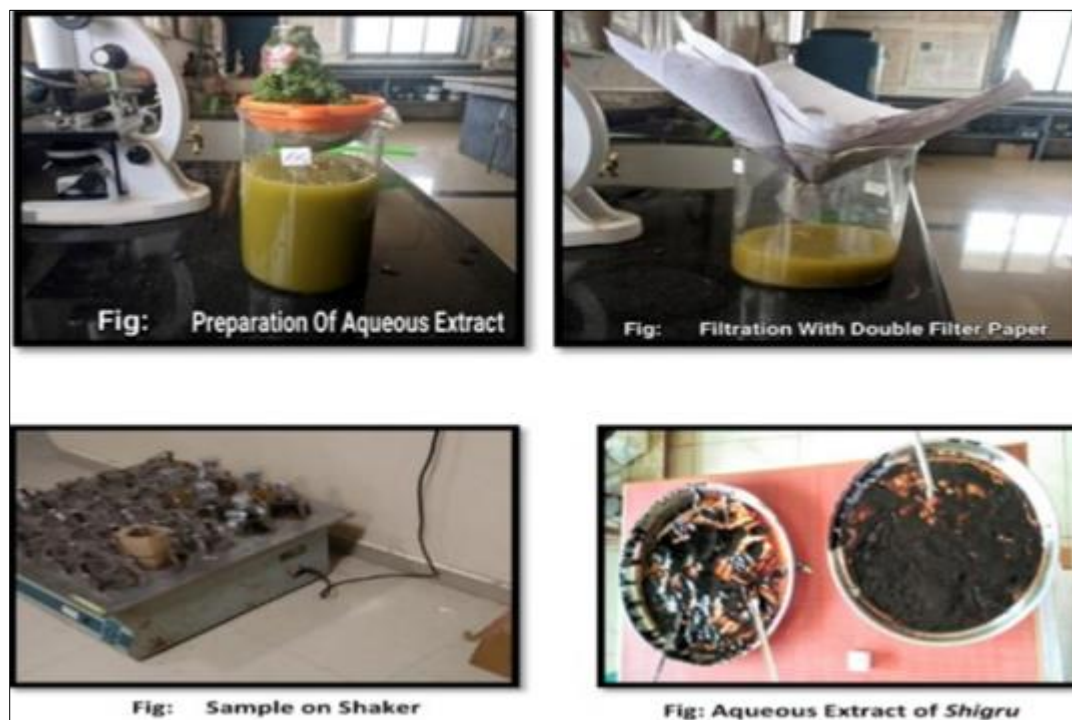


Figure 6 Preparation of Aqueous Extract

Preparation of 10% aqueous extract ⁽¹⁰⁾

- 10 g powder of aqueous extract of *Moringa oleifera* Lam leaves was steeped in 100 ml of distilled water and
- Filtered through a muslin cloth followed by filter paper (No.1. Whatman).
- After 24 h of soaking at room temperature (i.e., 22 °C) the extract was used for further study. As adopted from Singh et.al. (1989).

4 Finished product standardization

Table 2 Phytochemical Screening of *Shigru* Extract

Test for carbohydrate	Present	Complies the test
Test for Protein	Present	Complies the test
Test for alkaloids	Present	Complies the test
Test for anthraquinones	Absent	Doesn't Complies the test
Test for tannins	Present	Complies the test
Test for flavonoids	Absent	Doesn't Complies the test
Test for saponins	Present	Complies the test
Test for steroids	Present	Complies the test
Test for terpenoids	Present	Complies the test

Table 3 Physico-chemical, Heavy metal, Microbial contamination, Phytochemical Analysis

Test	Result
PH	4.84
Moisture content	11.6697% w/w
Alcohol Soluble extractive	26.24%
Water Soluble Extractive	86.64%
Heavy Metals	
Lead	Complies
Arsenic	Complies
Mercury	Complies
Cadmium	Complies
Microbial contamination	
Total microbial count	76 cfu/g
Total yeast & moulds counts	<10 cfu/g
E. coli	
Salmonella species/g	Absent/g
S. aureus	Absent/g
Pseudomonas aeruginosa	Absent/g
	Absent/g
Determination of alkaloids	3.524%
Determination of flavonoids	3.84%
Determination of saponins	1.752%
Determination of tannins	9.36%

5 HPTLC Fingerprint Profile: ⁽¹¹⁾

HPTLC is a planar chromatography where the separation of sample components can be achieved on high performance layers with detection and data acquisition using an advanced workstation.

Table 4 HPTLC analysis

Stationary Phase	HPTLC Precoated, Silica gel 60, F ₂₅₄ (Merck KGaA)
Thickness	0.2 mm
Mode of application	Band
Sample applicator	Linomat 5 (Semiautomatic Applicator)
Band width	6 mm
Solvent front pos.	85.0 mm
Solvent volume	10.0 ml
Drying device	Oven
Slit dimension	8.00 × 0.90mm, Macro
Scanning speed	20 mm/s

Data resolution	100 μm /step
Visualization At	Through UV-Cabinet under 254 nm & 366 nm and under Day light also.
Post Chromatographic	Vanilin sulphuric acid. Anisaldehyde Sulphuric acid (Only Derivatization for 6-Gingerol)
Measurement mode	UV absorbance / reflectance
Separation technique	Ascending
Scanning mode	Single level
Sample applied	6 μl , 4 μl

5.1 Observation

HPTLC plate of aqueous extract of leaves of *Moringa oleifera* scanned at different wavelength such as 254 nm wavelength, 366 nm wavelength and 540 nm wavelength which showed the presence of various phytochemical constituents at different ranging of Rf values.

5.2 In vitro study

In vitro study (assessment of anticancer activity of leaves of *Moringa oleifera* Lam.)

5.3 Cell line of oral cancer used for the study:

The in vitro study was carried out on the given cell line i.e., SCC 40, and SCC 29B by using aqueous extract of Shigru leaves.

5.4 Method of in vitro anti-cancer study

The cytotoxicity is checked by using SRB assay. The general purpose of SRB assay is to measure viable cells in relatively high throughput (96: well plates) without the need for elaborate cell counting.

5.5 Schematic presentation of in vitro testing

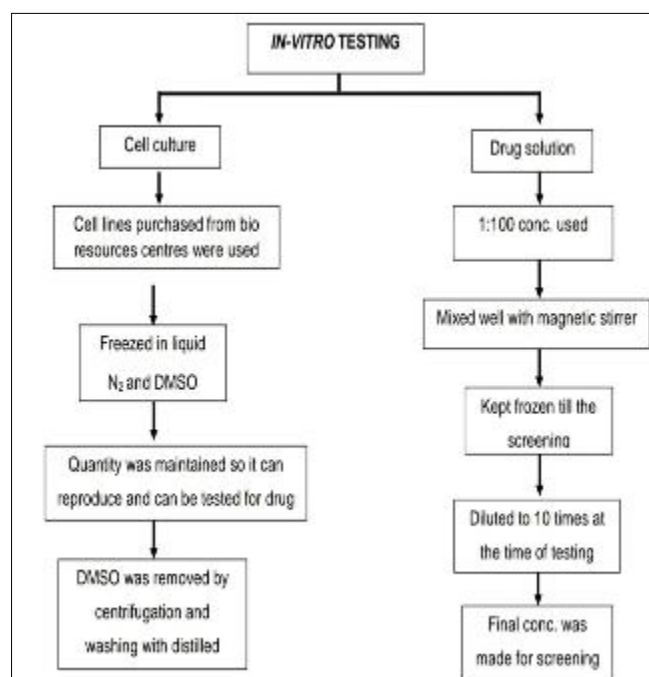


Figure 7 Schematic presentation of *in vitro* testing

6 Observation of drug concentration SCC-40 cell line

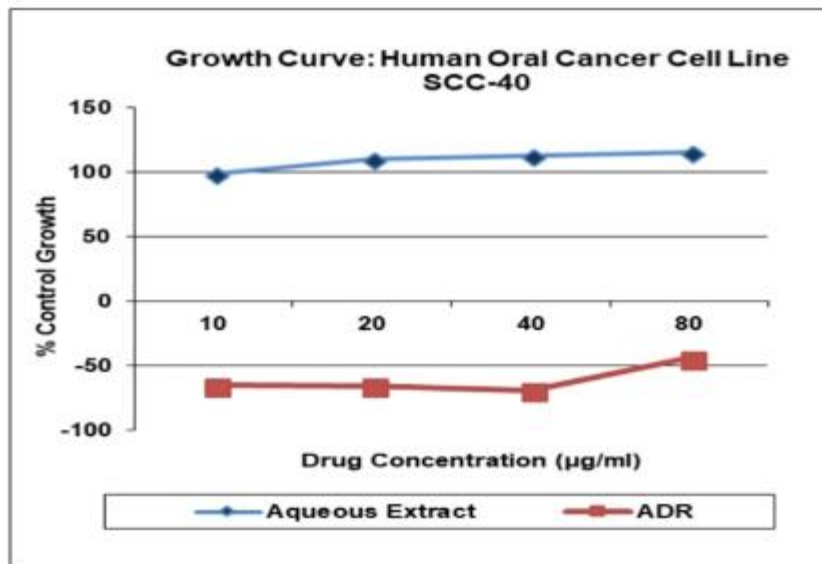
Table 5 Effect on SCC:40 Cell line of Oral Cancer

1.00	Human Oral Cancer Cell Line SCC:40															
% Control Growth																
Drug Concentrations (µg/ml)																
Experiment 1				Experiment 2				Experiment 3				Average Values				
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
Aqueous Extract	98.7	103.3	107.6	114.2	102.3	109.8	114.8	115.5	95.7	118.3	115.5	115.6	98.9	110.5	112.6	115.1
ADR	:63.9	:68.0	:69.4	:45.3	:61.5	:58.4	:64.0	:35.7	:70.2	:70.3	:74.1	:51.6	:65.2	:65.6	:69.2	:44.2

Drug concentrations (µg/ml) calculated from graph

SCC:40	LC50	TGI	GI50*
Aqueous Extract	NE	NE	NE
ADR	NE	<10	<10

Graph No.1 Growth curve: Human oral cancer cell line SCC-40



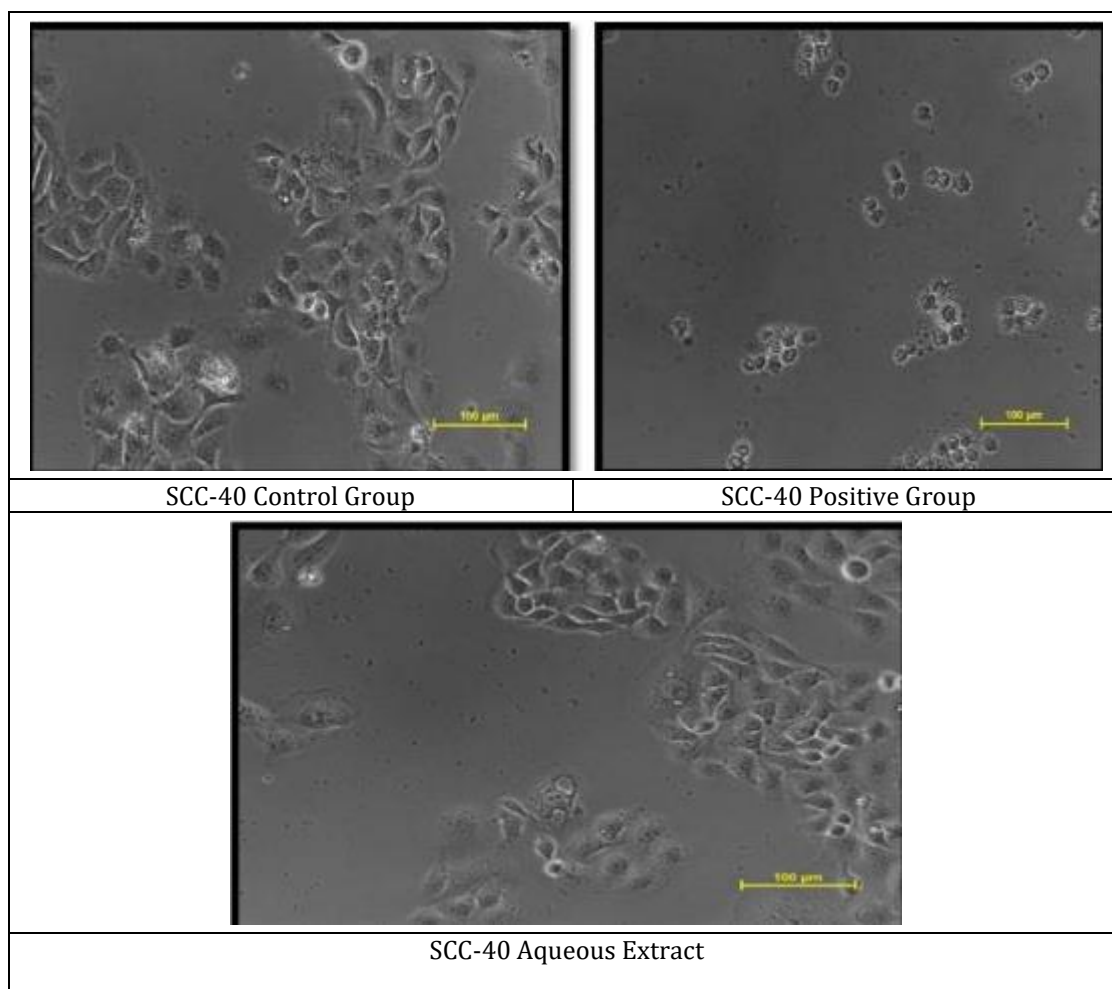


Figure 8 Photographs Showing effect of extract on SCC 40 Cell line

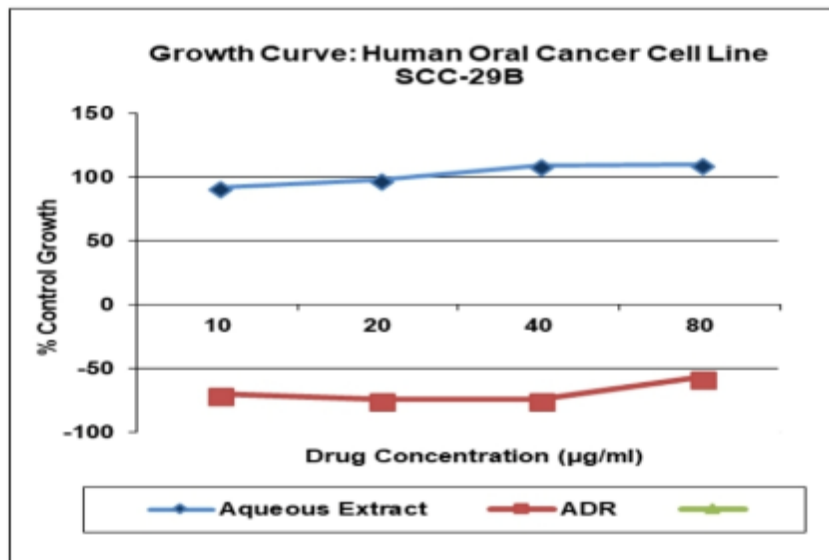
7 Observation of drug concentration scc-29 b cell line

Table 6 Effect on SCC:29B Cell line of Oral Cancer

Human Oral Cancer Cell Line SCC:29B																
% Control Growth																
Drug Concentrations (µg/ml)																
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
Aqueous Extract	85.0	95.4	112.0	105.0	96.3	98.6	102.9	108.1	93.2	98.8	110.6	115.7	91.5	97.6	108.5	110.9.6
ADR	:71.0	:74.4	:75.3	:61.6	:71.5	:74.2	:72.8	:52.5	:67.7	:75.2	:75.5	:57.0	:70.1	:74.6	:74.6	:57.0

Drug concentrations (µg/ml) calculated from graph			
SCC:29B	LC50	TGI	GI50*
Aqueous Extract	NE	NE	NE
ADR	<10	<10	<10

Graph No.2 Growth curve: Human oral cancer cell line SCC-29B



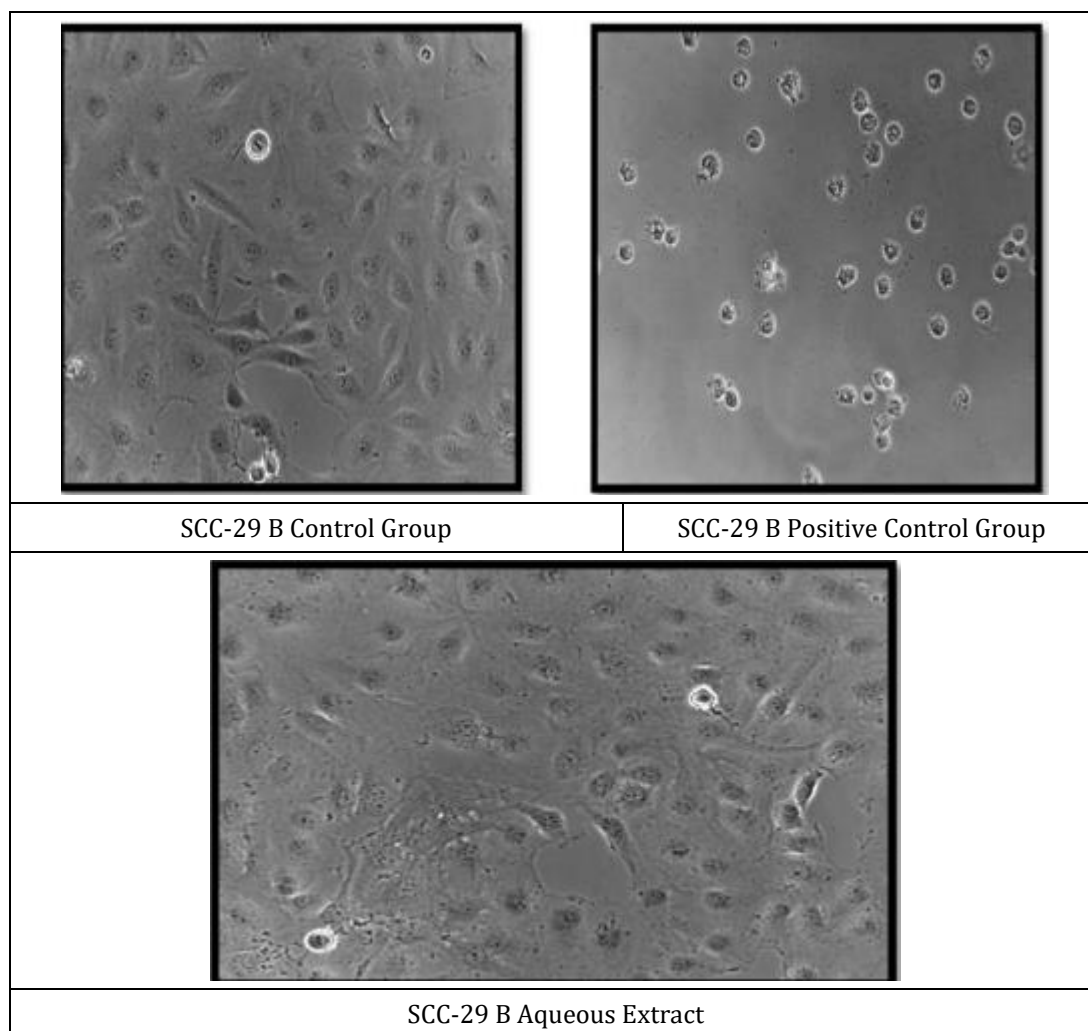


Figure 9 Photographs Showing effect of extract on SCC 29B Cell line

Observation

7.1 Effect of aqueous extract of leaves of *Moringa oleifera* on SCC-40 cell line of oral cancer

According to Table No:1 and graph no. 1 the average values at 10 μ g/ml, 20 μ g/ml, 40 μ g/ml, 80 μ g/ml are > 20 μ g/ml and no Inclination in graph was seen. These results indicate the test drug (i.e., aqueous extract of *Moringa oleifera* Lam) fail to show anticancer activity on the cell line SCC-40 of oral cancer.

7.2 Effect of aqueous extract of leaves of *Moringa oleifera* on SCC-29 B cell line of oral cancer

According to Table No: 2 and graph no. 2 the average values at 10 μ g/ml, 20 μ g/ml, 40 μ g/ml, 80 μ g/ml are > 20 μ g/ml and no Inclination in graph was seen. These results indicate the test drug (i.e., aqueous extract of *Moringa oleifera* Lam) is inactive to show anticancer activity on the cell line SCC-40 of oral cancer.

8 Result

There is no effect of 10% aqueous extract of leaves of Shigru (*Moringa oleifera* lam.) on SCC-29 B cell line and SCC-40 cell lines of oral cancer. As no matter how we increased the concentration level from 10 μ g/ml, 20 μ g/ml, 40 μ g/ml to 80 μ g/ml., we found that the increase in the percentage of molecules of 10% aqueous extract in the drug concentration well, the rate of cell growth did not decrease.

9 Discussion

The test drug proved standard by comparing all API standard values by doing various standard test such as pharmacogenetic, Physicochemical, phytochemical, Heavy metal test, Microbial contamination.

As per result obtained from SCC-40 & SCC-29B oral cell line, the concentration of the extract was increased up to 80 µg/ml. Adriamycin was used as control group. Bar diagram is plotted based on average value obtained from each experiment Which denotes the effect of drug and control drug on selected cancer cell line. The results can be discussed of anticancer study on oral cell lines SCC-40 & SCC-29B were as follows:

Statistical analysis of various concentration from 10 µg/ml to 80µg/ml doesn't shows significant anti- cancerous action i.e., Cytotoxic activity on SCC-40 & SCC-29B oral cell lines. Study drug was taken in 4 concentrations i.e., 10 µg/ml, 20µg/ml, 40µg/ml, & 80µg/ml. Each was compared to the Standard drug Adriamycin. As the concentration of drug was increased from 10 µg/ml to 80µg/ml there was no cytotoxic effect seen as compared to standard drug. The above observation suggests that there is no cytotoxic activity of 10% Aqueous extract of *Shigru* leaves on Oral cell line cancer.

10 Conclusion

- The phytochemical screening for aqueous extract of *Shigru* (*Moringa oleifera* Lam) leaves showed the presence of alkaloids, Saponins, glycosides, steroids, terpenoids, tannins, carbohydrates and proteins. But the extract does not content flavonoids and Anthraquinones.
- Flavonoids and anthraquinones are the phytoconstituents mainly responsible for anticancer action.
- The extractive values show higher values for alcohol soluble extract as compared to the water-soluble extracts.
- In SRB ASSAY protocol though we increased the concentration level from 10µg/ml, 20µg/ml, 40µg/ml to 80µg/ml., we found that the increase in the percentage of molecules of 10% aqueous extract in the drug concentration well, the rate of cell growth did not decrease. From this observation we have concluded that how many times we increase the concentration of aqueous extract, it has a limited scope for its anticancer activity. We think that the Cause behind this phenomenon is that the water-soluble portion of the *Shigru* (*Moringa oleifera* Lam) leaves have inadequate properties to come over the cell propagation in the cancerous growth.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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