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Wound healing potential of chloroform extract of leaf and fruit of *Morinda pubescens* – An *in silico* and *in vitro* approach

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ABSTRACT

Genus *Morinda* grows in the wild areas and extensively distributed over southern India. In India, many species have been found available, of which *Morinda pubescens* (Synonym: *Morinda tinctoria*) predominantly grows as a weed tree in vacant agricultural land. It has been cultivated in different parts of Tamilnadu state of India. It is widely used in the Indian traditional system of medicine. Based on the folklore claim Insilico and Invitro studies were performed using several methods. Docking experiment was carried on with iGemdock, docking software to initially study the energy binding values of constituents. Having studied the various experiments, Glycogen synthase kinase and several MMP's [1] was used as a protein. Energy binding values showed up prompt results which indicate the affinity of the molecules towards the protein. Plant extract at three different concentrations was taken and MTT assay was performed. Then, with the help of the C2C12 cell line which is a muscle cell line, we evaluated the effect of our plant extract on the cells. Among the chloroform extract of leaf (100µg/ml) and fruit (100µg/ml), fruit extract was found to have the efficiency to fill the gaps between cells with newer ones and better elongation of cells compared to control (Nutrient treated).

Keywords: *Morinda pubescens*, iGemdock, Glycogen synthase kinase, MMP's, MTT, C2C12.

INTRODUCTION

Wound healing is a restorative response by the tissue during injury. It involves the complex cascade of cellular events that includes three faces, namely resurfacing, reconstitution, and restoration of the tensile strength of injured skin. Often healing process is explained in four terms with each classic phase overlapping each other: Hemostasis, inflammation, proliferation and maturation. During clot formation platelets play the crucial role. In our experiment, the docking simulation is done initially with MMP's model and cell line study with the help of a muscle cell line was performed by scratch assay [2]. Choosing the target (Protein) for the ligands (Constituents) should be specific. With MMP's model we collected the four fibrous structural proteins that is responsible for the healing the extra cellular matrix against the tissue repair. All four constituents were compared with each other to analyze the molecule with the most binding energy value. The constituents of the plant were already reported [8] and we retrieved them protein data bank.

Similarly the cell line study was also performed with muscle cell line. Initially the cells were purchased from NCCS (National centre for the cell sciences). With the help of the chemical study we arrived at the results that both the leaf and fruit had the phytochemicals that are responsible for the healing process. After the optimization of the dose, it was carried onto the lab for incorporation into cell lines for the identification of the closure of wounds. With having the nutrient treated cell line as control, extract of leaf and fruit were compared.

MATERIALS AND METHODS

In-silico method

Docking simulation was done by using iGemdock software and the conversions of the compounds into .pdb format were made by the software openbabel [3]. With the help of the earlier study about the isolation of the constituents [4], the structure of the compound were collected the protein data bank. All the constituents and proteins were separately categorized. The MMPS's and protein kinase were also collected from RSCB protein data bank, namely: collagenase, gelatinase, elastase and glycogen synthase kinase.

Once the ligand and protein are set, the binding site preparation was done and the ligands were loaded. Initially with basic scoring functions and the default inputs, the outpath was located for the docking results to be stored. Having parameters set for the docking, the process was started. During the proceeding, software itself aligns the receptor and ligand for the best pose and generates the value for the binding energy, van der waals force value, hydrogen bonds and average connecting pairs. When the docking is over, display of the docked pose were projected and the best pose with the visual of both the receptor and ligand were identified and edited. The, corrected image was exported as .GIF into a folder for the review purpose.

Collection and preparation of plant extract

The leaves and fruit of *Morinda pubescens* (Figure 1 & 2) were collected from nearby MGR University, Maduravoyil, Chennai, Tamil Nadu, India, in the month of June 2016. The Plant material was identified and authenticated by Prof. P. Jayaraman, Ph.D., Retired Professor, Presidency College, Tambaram, Chennai. The collected plant material was free from diseases and also free from contamination of other plants.

Figure 1: Fruit of *Morinda pubescens*

Figure 2: Leaf of *Morinda pubescens*

Freshly collected plant material was dried in shade and then coarsely powdered in a blender. The coarse powder was successively extracted in an aspirated bottle with Petroleum ether and chloroform for about 72, 48, 24 hours by maceration technique. Among the extract, chloroform was taken after decantation, filtered through the whatmann filter paper no: 41. Solvent was distilled and concentrated. The concentrated extract was used for further studies.

***In-vitro* method**

Purchased cell lines were cultured for the *in vitro* study. Primary isolations from tissue or blood were often used for the wound healing assays. For every successful assay, the cell culture condition must be reproducible for stable phenotypes. Culturing protocols can be obtained from ATCC or NCCS for most cell lines. They include details such as, subculturing, growth medium and expected doubling time.

Wound healing assays are carried out with a thin layer of cells grown on a plastic or glass substrate. Almost all the epithelial and endothelial cells, thin layers of cells are easily attained because the cells form confluent mono layers. The underlying substrate and the associated extra cellular matrix (ECM) are also important considerations in the wound healing assay. Some cell types can grow directly on plastic or glass substrates, whilst others require a coating of extracellular matrix components such as gelatin, collagen, or fibronectin in order to cling.

Cell culture preparation protocol

The obtained cells were cultured in the DMEM (Dulbeccos's Modified Eagle Medium) containing 10% FBS, 4.5g of Glucose, 20mM Glutamine, 5% Penicillin and streptomycin were used for the culturing of C2C12 cells. The cultured cells were incubated for 48 hours in the T-75 culture flask. The culturing condition was followed as per the suggestions provided by ATCC After 48hrs of incubation, the cells are seeded for proliferation/cytotoxicity assay at the cell density of $6X10^3$ per well in 96 well sterile tissue culture plate. Different groups have been specifically marked with respect to the chloroform extract of leaves, fruits and control groups.

In major in-vitro assays of cell's response regarding population to external factors is merely based on the cell viability and measurement of proliferation. MTT which is chemically known as (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide). In this assay, the active cells are expected to turn the yellow tetrozolium to formazan, a purple color complex. The resulting intracellular formazan complex is solubilized using DMSO and quantified spectrophotometrically at 570nm.

Aim of MTT assay is to ascertain the cell proliferation rate and conversely, if metabolic events lead to apoptosis or necrosis, there will be reduction in the cell viability. The absence of viable cells is determined by the low background absorbance values for the reagent.

MTT Assay [5]

- The cultured cells in 96 well plate were incubated for 48 hours along with the different concentration of chloroform extract of leaf (50, 100, 250 µg/ml), fruit (50, 100, 250 µg/ml) or vehicle control (0.2% DMSO).
- After incubation, the medium was removed and 100 µL of MTT (0.5mg/ml) reagent prepared in PBS was added to each wells and incubated for 3 hours.
- After three hours, the presence of purple color complex was observed which indicated the formation of formazan in respective groups of cells and the MTT reagent was completely removed all the wells.
- The intracellular formazan crystals were dissolved by addition of 100µl of DMSO in all the wells for 15 minutes.

- The concentration of the formazan in each well was measured under the multi well plate reader at 570nm and 630nm respectively.

In scratch assay, the cells monolayer is scratched evenly in the centre of which leaves a gap in between the cells and the activity is determined with respect to the growth of cells towards each other. Before the treatment with the plant extract, the floating cells were washed off. Objective is to check whether the extract the cell elongation between the scratched surfaces compared to nutrient treated cells.

Scratch assay procedure

- In this experiment we used skeletal muscle C2C12 cell line.
- Scratch was made in the cell line using a 10µl microtip in each well and the floating cells were washed using PBS.
- Cells were added with the respective concentration (100µg/ml) which was selected based on the MTT assay in accordance with the fruit and leaf chloroform extract. Vehicle control of 0.2% DMSO was used.
- The well plate before (0hr) and after administration (24hr) of the extract was photographed using Inverted light microscope.
- The cells which are proliferated and elongated into the scratched area were observed between the gaps.

RESULTS AND DISCUSSION

Docking reports

Docking studies have shown some significant values that our phytoconstituents have considerable binding energy. Among all, stigmasterol and urosolic acid has unveiled higher binding energy equally. Receptors were chosen based on a reason, since fibrous structural proteins and protein kinase are majorly responsible for the wound healing. Nitrofurazone was used as a standard drug to compare with the marketed product. All the values were negative, which indicates that the affinity of compounds towards the receptor is good.

Listed tables show the results for its respective receptors and their affinity with the ligands. (From Table 1 – Table 4)

Table 1: Energy binding values of ligands with glycogen synthase kinase

Ligand	Total Binding Energy	VDW	HBond
Beto sitosterol	-72.8627	-70.6585	-2.20423
Stigmasterol	-70.344	-66.238	-4.10593
Taraxerol	-75.6924	-62.9467	-12.7457
Urosolic acid	-82.7107	-72.6744	-11.3415
Nitrofurazone	-94.3991	-57.0077	-34.3064

Table 2: Energy binding values of ligands with Collagenase

Ligand	Total Binding Energy	VDW	HBond
Beto sitosterol	-97.8217	-96.1372	-1.68454
Stigmasterol	-102.924	-100.424	-2.5
Taraxerol	-83.3931	-78.4045	-4.98858
Urosolic acid	-81.7395	-75.626	-6.7931
Nitrofurazone	-87.3455	-55.166	-31.2903

Table 3: Energy binding values of ligands with Elastase

Ligand	Total Binding Energy	VDW	HBond
Beto sitosterol	-43.351	-43.351	0
Stigmasterol	-51.7108	-51.7108	0
Taraxerol	-43.0218	-36.0218	-7
Urosolic acid	-47.329	-40.329	-7
Nitrofurazone	-2.16203	-2.16203	0

Table 4: Energy binding values of ligands with Gelatinase

Ligand	Total Binding Energy	VDW	HBond
Beto sitosterol	-87.7579	-85.4466	-2.31127
Stigmasterol	-88.0724	-78.5724	-9.5
Taraxerol	-85.2984	-85.2984	0
Urosolic acid	-90.039	-84.7526	-5.28638
Nitrofurazone	-71.6367	-50.8994	-20.7373

*VDW – Van der waals force
*HBond – Hydrogen bond

Images for the docked pose have been illustrated below; ligands have been highlighted in the image with pink color. (From Figure 3 – Figure 22)

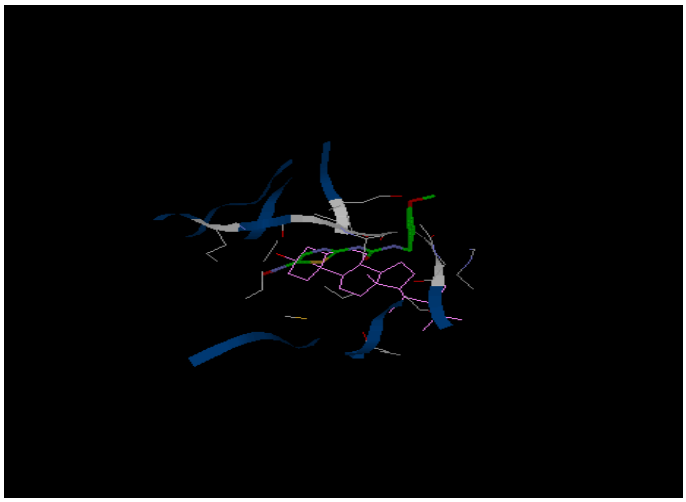


Figure 3: Beta sitosterol with Glycogen synthase kinase-3

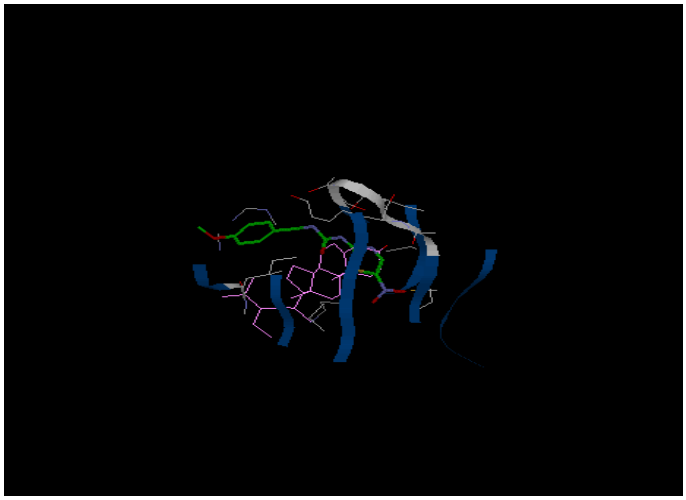


Figure 4: Stigmasterol with Glycogen synthase kinase-3

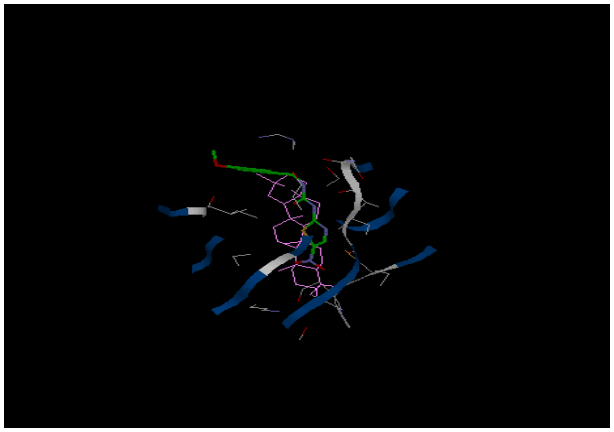


Figure 5: Taraxerol with Glycogen synthase kinase-3

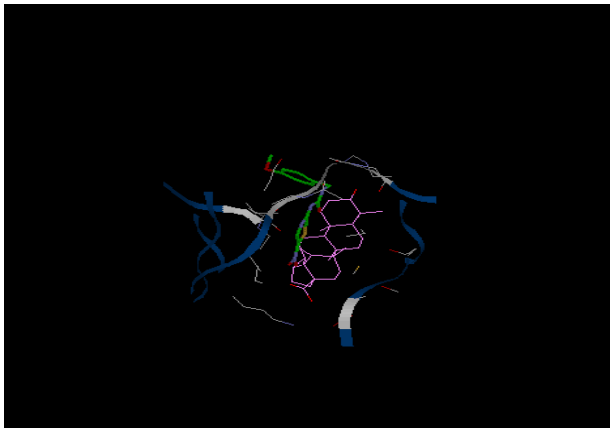


Figure 6: Urosolic acid with Glycogen synthase kinase-3

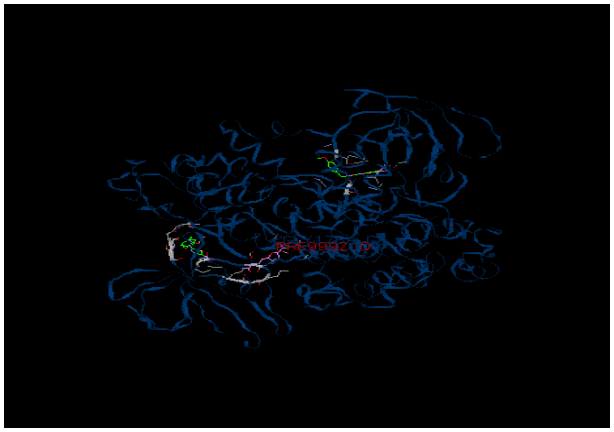


Figure 7: Nitrofurazone with Glycogen synthase kinase-3

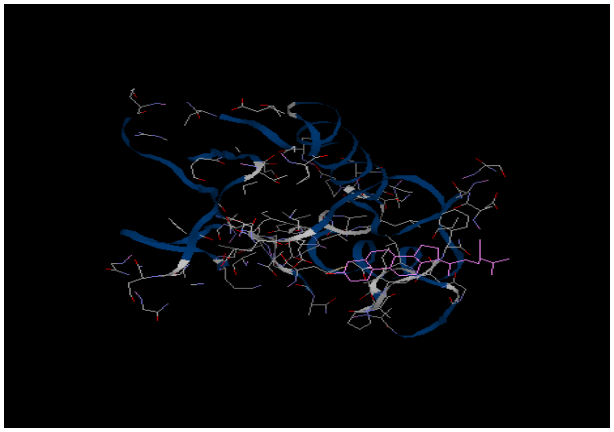


Figure 8: Beta sitosterol with collagenase

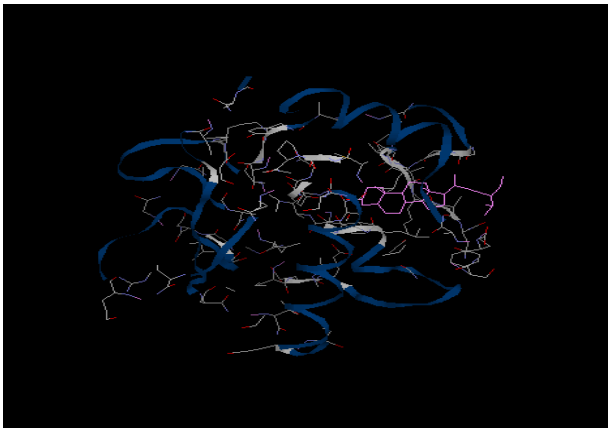


Figure 9: Stigmasterol with collagenase

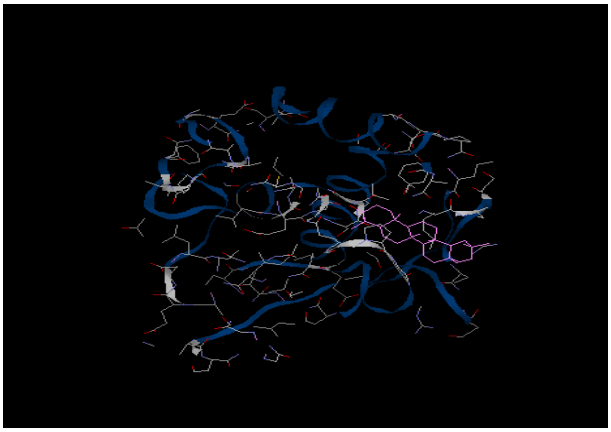


Figure 10: Taraxerol with collagenase

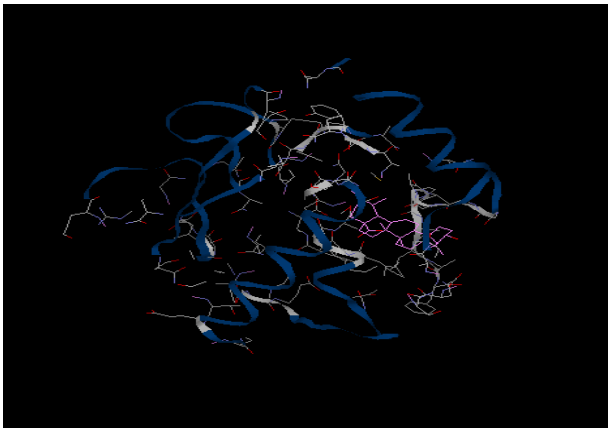


Figure 11: Urosolic acid with collagenase

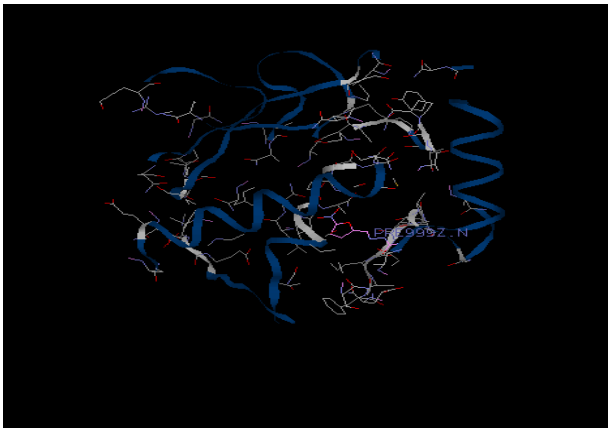


Figure 12: Nitrofurazone with collagenase

Figure 13: Beta sitosterol with elastase

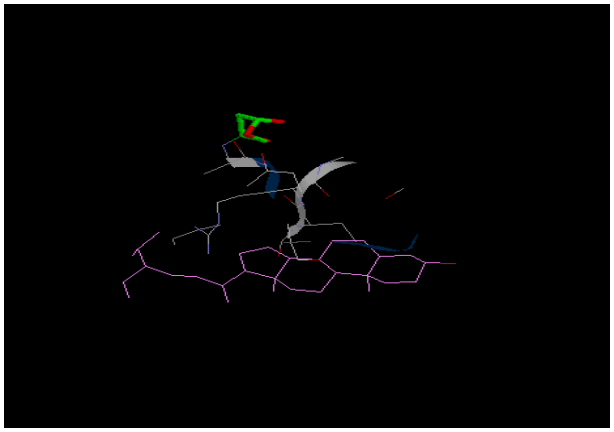


Figure 14: Stigmasterol with elastase

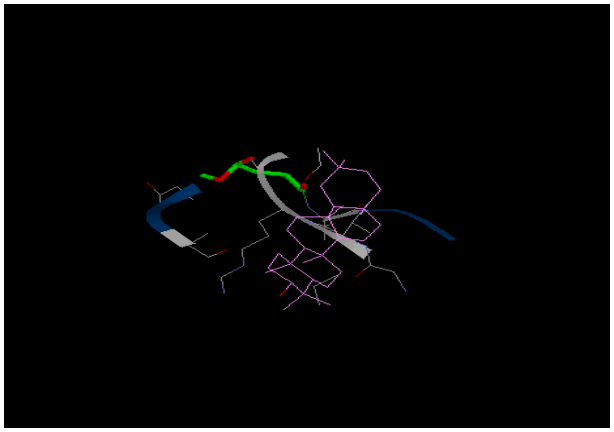


Figure 15: Taraxerol with elastase

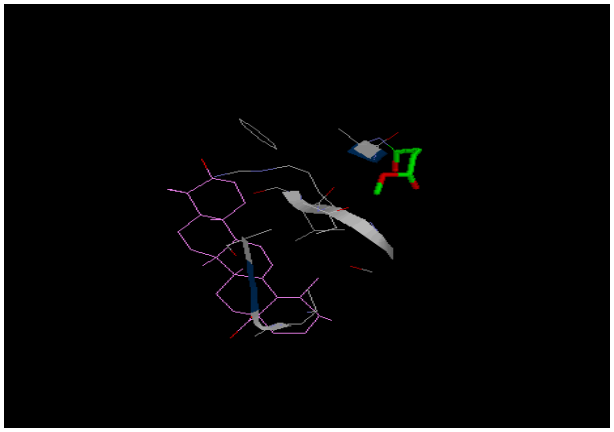


Figure 16: Urosolic acid with elastase

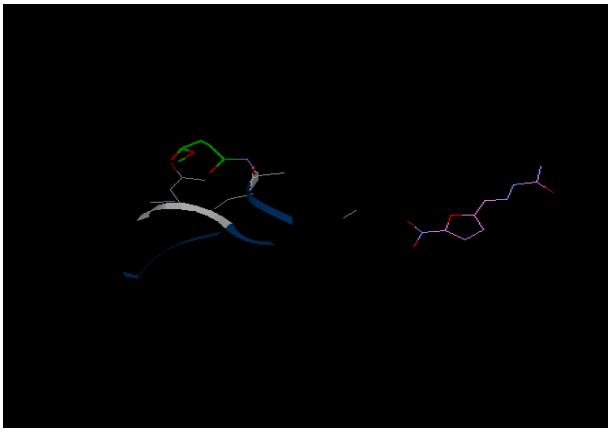


Figure 17: Nitrofurazone with elastase

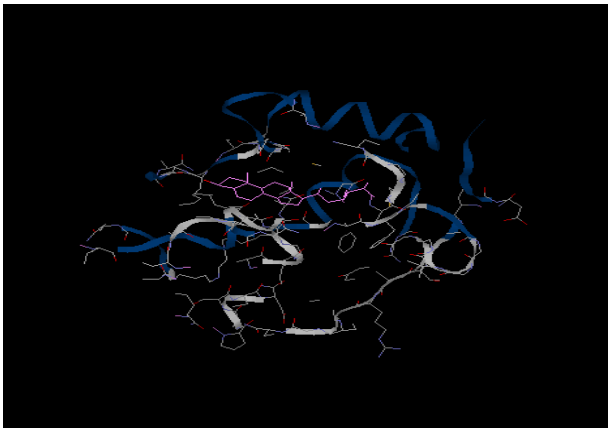


Figure 18: Beta sitosterol with gelatinase

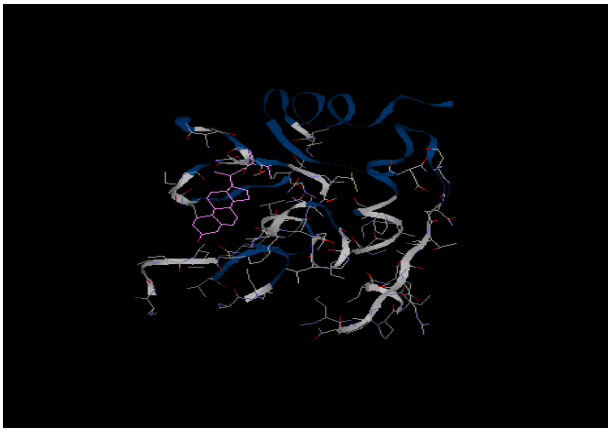


Figure 19: Stigmasterol with gelatinase

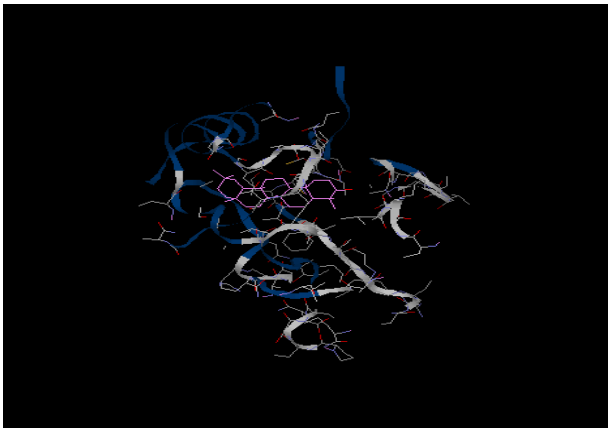


Figure 20: Taraxerol with gelatinase

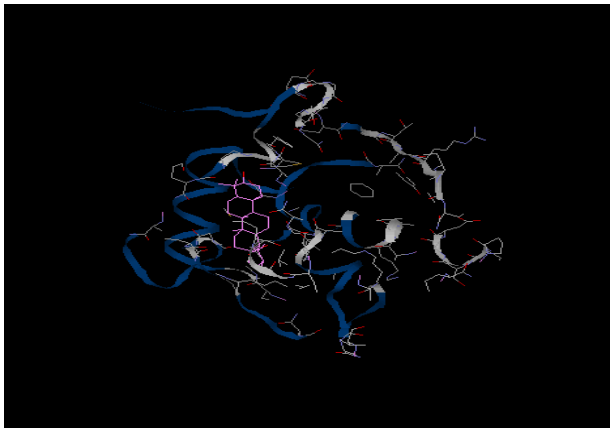


Figure 21: Urosolic acid with gelatinase

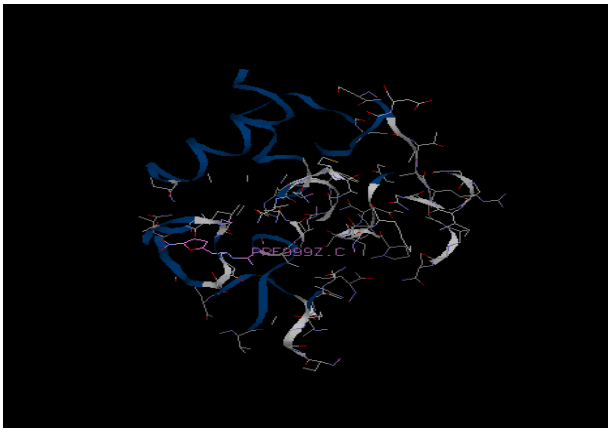


Figure 22: Nitrofurazone with gelatinase

Results clearly show that ligands were able to provide a strong affinity towards receptor. In some cases, ligand has shown up more affinity when compared to standard. Thus, our phytoconstituents can be used for wound healing assay in future for a cost effective approach.

MTT assay reports

Plant extract dose was fixed using the MTT assay; initially three rough concentrations were taken and studied. It was tested with two samples in order to arrive at the average. Spectrometrically it was observed at two concentrations; 570nm & 630nm. Among which the 630 nm was observed to eliminate the absorbance showed by the reagent itself. Average of two samples OD was calculated. With arrived values, the %proliferation was calculated using the formula;

$$\% \text{ Proliferation} = \frac{\text{Sample OD}}{\text{Vehicle control OD}} \text{ X } 100$$

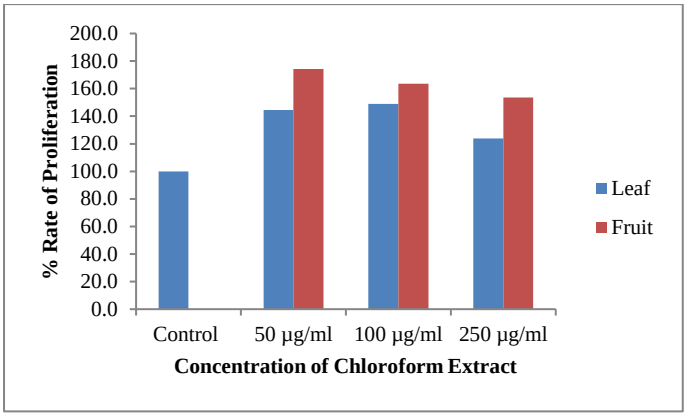
Table 5: Multi well plate readings

Groups	Conc (µg/ml)	Sample 1 OD		Final OD	Sample 2 OD		Final OD
		570nm	630nm	(570 - 630)	570nm	630nm	(570 - 630)
Control	0.2% DMSO	0.957	0.570	0.387	0.982	0.582	0.400
Leaf	50	1.441	0.858	0.583	1.448	0.894	0.554
	100	1.448	0.870	0.578	1.466	0.872	0.594
	250	1.245	0.762	0.483	1.294	0.802	0.492
Fruit	50	1.24	0.725	0.515	2.082	1.225	0.857
	100	1.616	0.958	0.658	1.544	0.914	0.630
	250	1.453	0.859	0.594	1.486	0.872	0.614

Table 6: % Rate of Proliferation

Groups	Conc (µg/ml)	Sample 1 (Final OD)	Sample 2 (Final OD)	AVERAGE	% Rate of Proliferation
Control	0.2% DMSO	0.387	0.400	0.394	100.0
Leaf	50	0.583	0.554	0.569	144.5
	100	0.578	0.594	0.586	148.9
	250	0.483	0.492	0.488	123.9
Fruit	50	0.515	0.857	0.686	174.3
	100	0.658	0.630	0.644	163.7
	250	0.594	0.614	0.604	153.5

The result shown significantly proves the above chosen concentrations of chloroform extract of fruits and leaves were not toxic to the C2C12 cells. Values obtained for the leaf and fruit extract shows increased percentage proliferation rate when compared to the control (Table 5 & 6). This preliminarily study proves that the extracts enhance the proliferation of C2C12 cells. The fruit extracts have shown efficient and increased rate of proliferation of C2C12 cells than the leaf extract. Based on the results of the present study, the 100 µg/ml concentration of fruit or leaf extract was selected for further evaluation.



Graph 1: Concentration (µg/ml) Vs % proliferation

Scratch assay reports

In scratch assay the middle concentration of 100 µg/ml of extract were added to cells. With the help of the inverted light microscope, the photographs of the cells were taken at 0th hour and 24th hour respectively. As the cells start to migrate and elongate with the help of the plant extract and was compared to nutrient treated cells respectively.

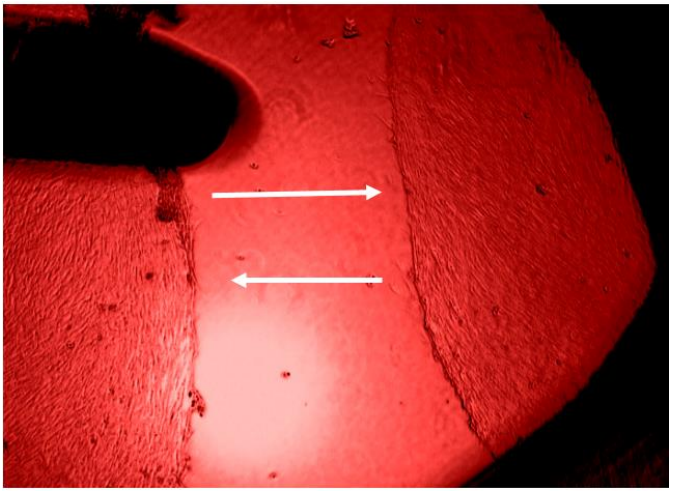


Figure 23: Control (0th hour)

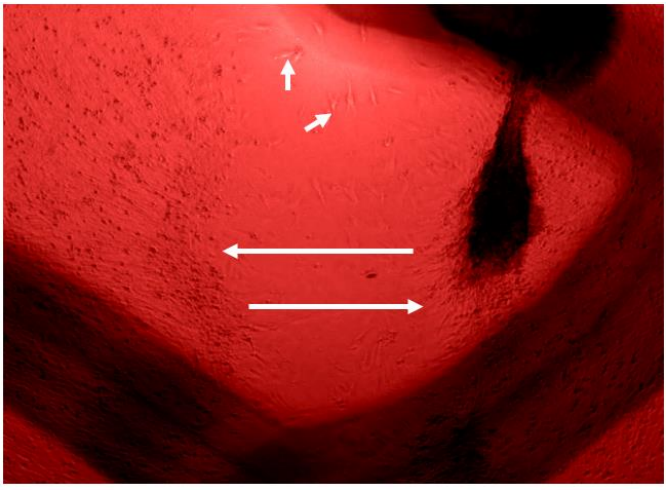


Figure 24: Control (24th hour)

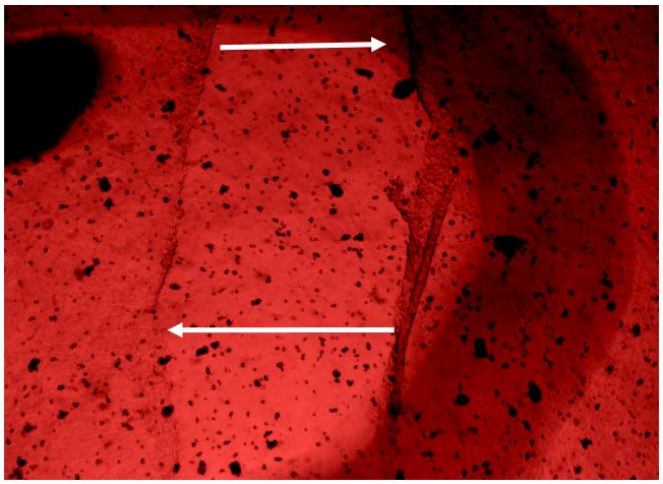


Figure 25: Chloroform extract of leaf (100µg/ml) at 0th hour

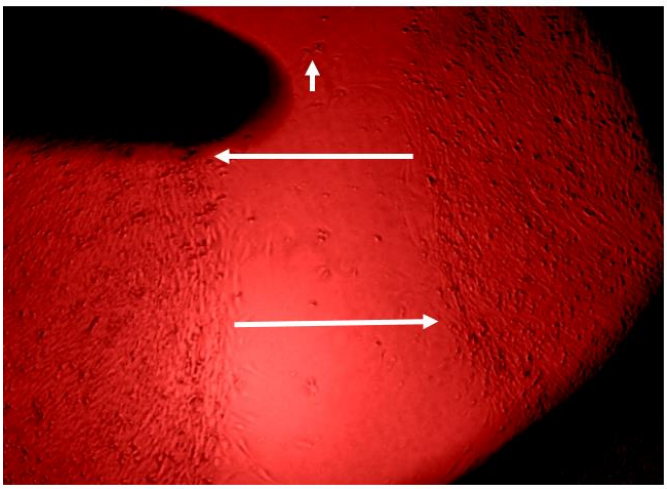


Figure 26: Chloroform extract of leaf at 24th hour

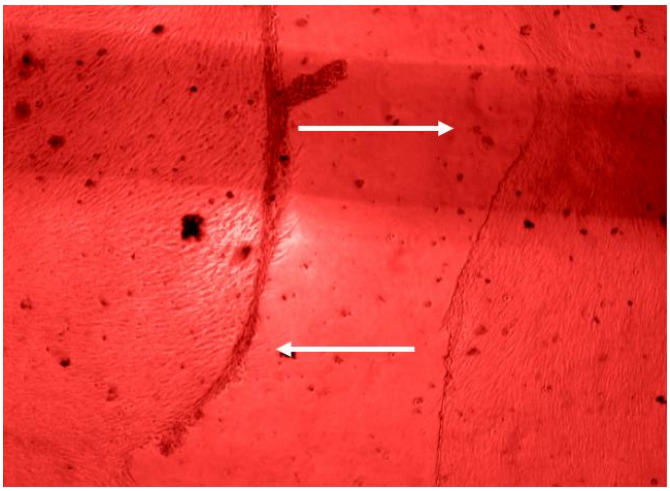


Figure 27: Chloroform extract of fruit (100µg/ml at 0th hour

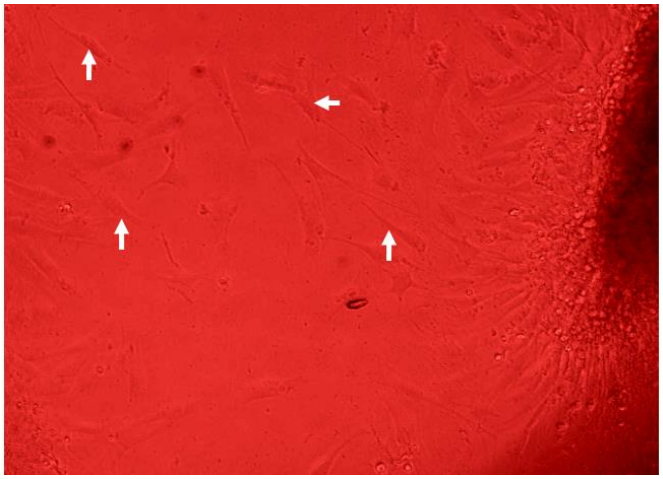


Figure 28: Chloroform extract of fruit (100µg/ml) at 24th hour

From the above represented figures of the different extract at different hour showed a significant cell growth when compared to the control (no drug). The chloroform extract of fruit has shown a better cell elongation in the figure 28 when compared to the similar figure taken at the 24th hour of the control. The chloroform extract of leaf has also shown a significant cell elongation when compared to the standard in the figure 26 for the reference. There are growths in the control as well as seen in figure 24 and way the cells have grown differs in the figure 26 and 28 respectively. Thus, this proves us the chloroform extract of the plant *Morinda pubescens* has wound healing activity. But the chloroform extract of fruit was considered to be effective when compared to the chloroform extract of leaf and control (no drug).

CONCLUSSION

Results obtained in the above study showed significant support that the plant has wound healing potential. It may be due to the capacity of itself to involve in the elongation of muscle cells. Thus, it can be treated for the deep cuts. With another experiment with the fibroblast cells, this can produce likely results. This will take this experiment even further isolation of the pharmacologically active constituents to comprehend the exact mechanism of action. Then purified chemical can be used to replace the conventional medicine in a cost effective way.

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