Combinatorial Therapies for Recurrent ER- Breast Cancers

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Introduction:

Cancers are the leading cause of mortality all over the world according to WHO. In this review, we aim to show the use of common Statins to have potential applications in combinatorial therapies. Disorders related to heart and blood vessels including coronary heart disease (interference in blood supply to heart muscles), cerebrovascular heart disease (interference in blood supply to brain), rheumatic heart disease, congenital heart diseases fall under CVDs. These disorders under acute conditions cause angina, strokes and fatal heart attacks, which caused the global death of 85% CVD patients in 2016 [1] [2]. This is related to high levels of cholesterol in plasma, since hypercholesterolemia is a primary risk factor of atherosclerosis and coronary artery disease [3].

Lovastatin is a naturally occurring statin drug, used for lowering cholesterol in those with hypercholesterolemia to reduce risk of cancer [4]. Pure Lovastatin is used in the manufacture of drugs, which is currently very expensive in the present market. The focus of this project is the extraction of statins from fruit and vegetable waste using filamentous fungi as literature survey has shown that these contain a fair amount of Statins. Fruits and vegetable peels being waste products are discarded anyway, and thus using these to extract statins will not only be cost effective but will provide better waste management solutions.

The prevalence of raised total cholesterol increases noticeably according to the income level of a country. In low-income countries around a quarter of adults had raised total cholesterol, in lower middle-income countries this rose to around a third of the population for both sexes. In high-income countries, over 50% of adults had raised total cholesterol; more than double the level of the low-income countries [5].

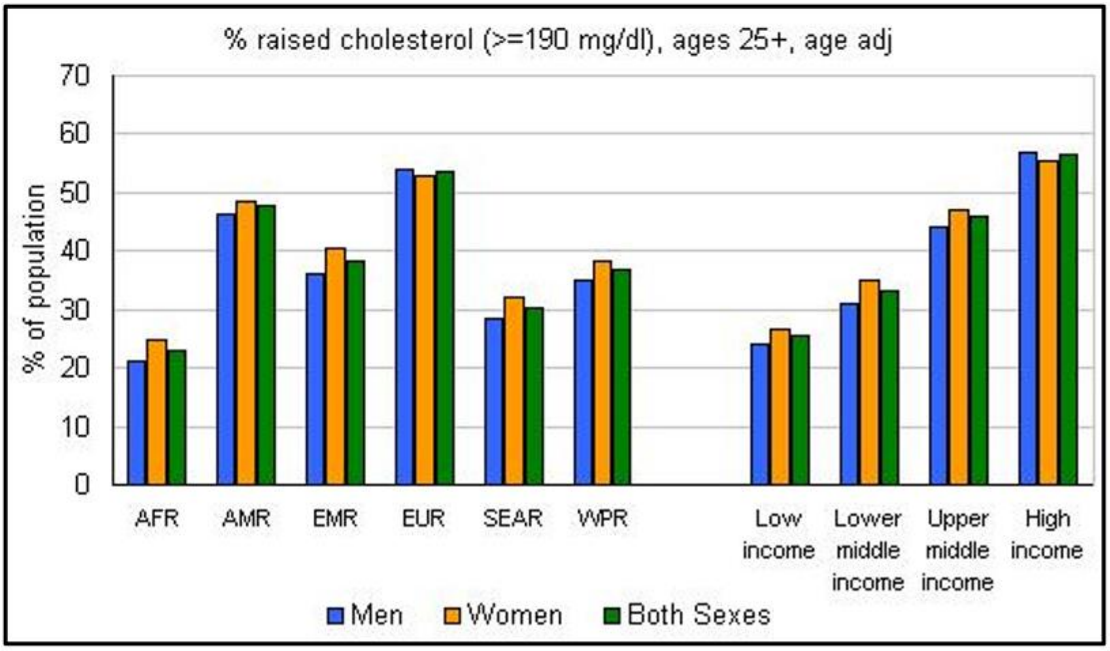


Fig1: % raised cholesterol in population [3]

As a rise in cholesterol level is one of the major concerns of the modern world, our team was motivated to carry out this project on statins. Statins are the primary class of drugs which are prescribed by practitioners over the world to lower cholesterol levels. However, they are not very economical for the poor thus we were driven to find an economical alternative for the production of the same.

The main objective of this research study was to carry out efficient extraction and characterization of lovastatin using organic waste. The food industry produces a large amount of organic waste, making it necessary to search for possible ways for their utilization. One way could be to use this 'waste' as a new and natural source of high-value functional ingredients due to the presence of bioactive compounds inorganic waste, which present health benefits.
Using bioactive molecules recovered from food waste as usable ingredients is a safe alternative to using food waste as an affordable source of useful compounds, while also helping to produce new food and drug products with health benefits and contributing to waste reduction management.

1. Statins:

Statins are a group of drugs that selectively inhibit hydroxymethylglutaryl coenzyme An (HMG-CoA) reductase, the regulatory and rate-limiting enzyme in cholesterol biosynthesis [6]. In this way, these compounds lower cholesterol; particularly low-density lipoprotein (LDL) or low density cholesterol ("bad cholesterol"); while slightly increasing high-density lipoprotein cholesterol ("good cholesterol"), thus, preventing plaque build-up inside the arteries.

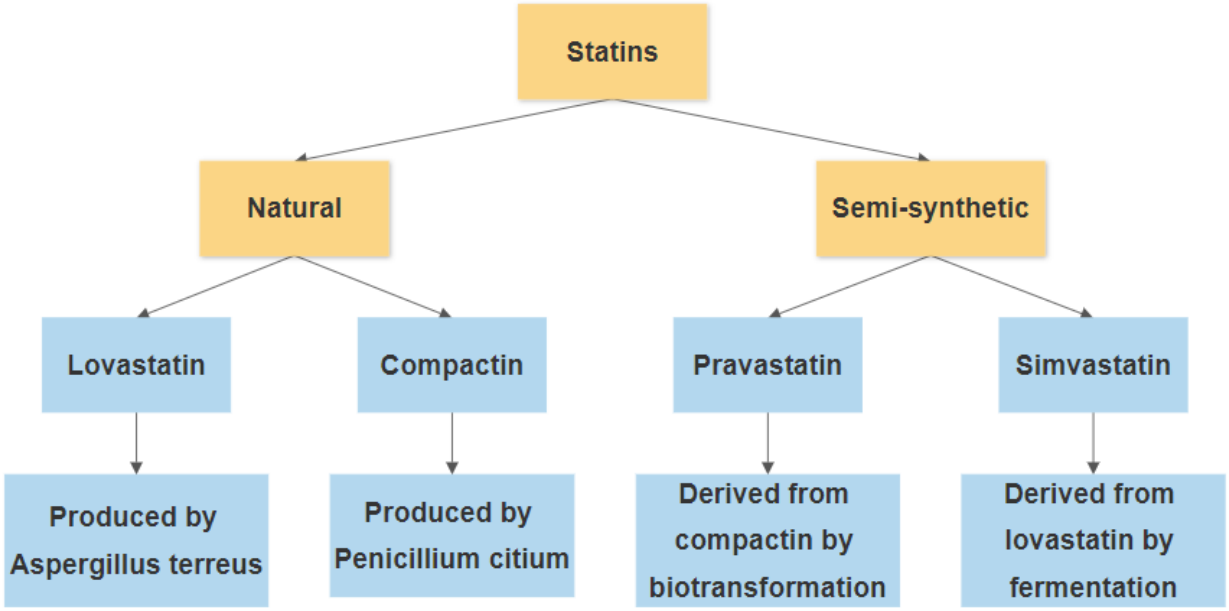


Fig 2: Classification of statins [7]

Simvastatin, the second leading statin in the market, is a lovastatin semisynthetic derivative. Lovastatin is a secondary metabolite produced by *Aspergillus terreus* strains by liquid submerged fermentation but can also be produced by the emerging technology of solid-state fermentation, which displays some advantages. In this report lovastatin has been extracted by solid state fermentation using organic waste as a substrate [8].

1.1 Structure and properties of Lovastatin

All statins possess a common structure, a hexahydro-naphthalene system and a ß-hydroxylactone; their differences are due to side chains (R1) and methyl groups (R2) around the ring [9].

Lovastatin is known to exist in open hydroxy acid as well as in lactone forms. The major form of lovastatin in fermentation broth is the open hydroxy acid form (Mevinolinic Acid). However, it is generally in lactone form (mevinolin) when administered to the patients as drug [10].

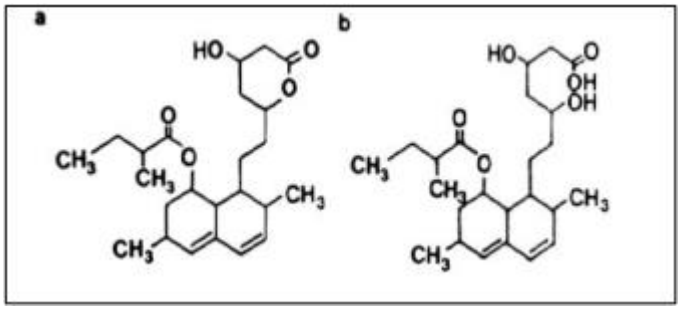


Fig 3: Structural formulae of (a) lactone; (b) open hydroxyl acid forms of lovastatin [11]

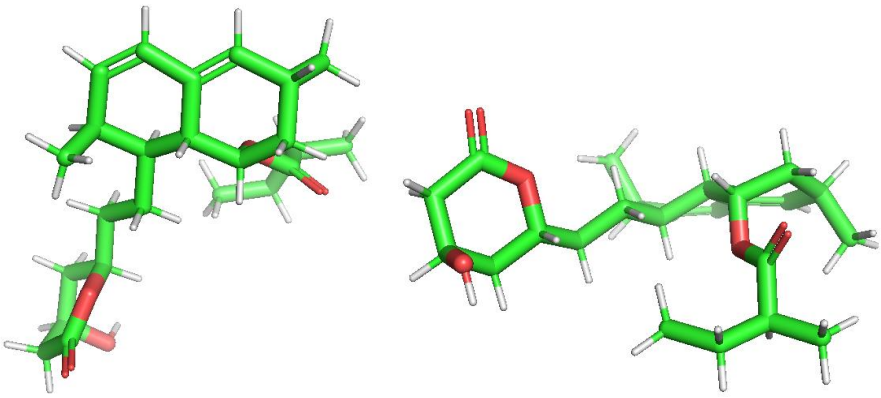


Fig 4: 3-D structure of lovastatin from pymol [7]

Table 1: Properties of lovastatin [from pubchem]

Property name	**Property value**
Molecular Mass	404.5 g/mol
Exact mass	404.256274 g/mol
Boiling point	559.2 ºC at 760 mmHg
Melting point	174.5 °C
Solubility in water	0.4X10-3 mg/mL at 25 °C

Well-closed, light-resistant containers at 5-30 °C are preferred to store lovastatin drugs. Under this storage method the stability tablets have a period of stability for 24 months from the date of manufacture and the tablets are light sensitive. When exposed to UV (approximately 3230 lux) or fluorescent (approximately 10,764 lux) light at 28 °C in air, the substance is stable for 24 hours or 1 month after being exposed to intense light conditions [12].

2. Materials and methods

Six different kinds of peels were taken to carry this out, three of which were fruits and three vegetables. Peels of potato, raw banana, onion, lemon, orange and pomegranate were obtained from daily kitchen waste, where fruits and vegetables were bought from local Bangalore markets. The peels were initially sun-dried for four days, and then completely dried in hot air oven at 50˚C for two days. The peels were then separately powdered and sieved though mesh numbers 12 and 22; to obtain particles in the range of 0.7-1.68 diameter. The powdered peels were taken in separate conical flasks. Enriched water including MgSO4.7H2O, NH4H2PO4 and NaCl was added to maintain 70% moisture content following which the flasks were autoclaved.

Aspergillus terreus spp. [13] was obtained on prepared slants from the Department of Microbiology and Biotechnology, Bangalore University, Bangalore. Potato Dextrose Agar (PDA) medium was prepared along

with Antibiotic (Ampicillin) to prevent any bacterial contamination. Spore suspension was added to each conical flask of powdered peels under sterile conditions to avoid contamination. These samples were then left for 15 days to obtain full fungal growth.

After solid state fermentation, the substrate was dried at 60˚C for 24 hours and then treated with Ethyl Acetate (pH 3). The samples are centrifuged, filtered. The supernatant was collected and vacuum dried at 50˚C for 20 mins. The dried sample is used for analysis and characterization.

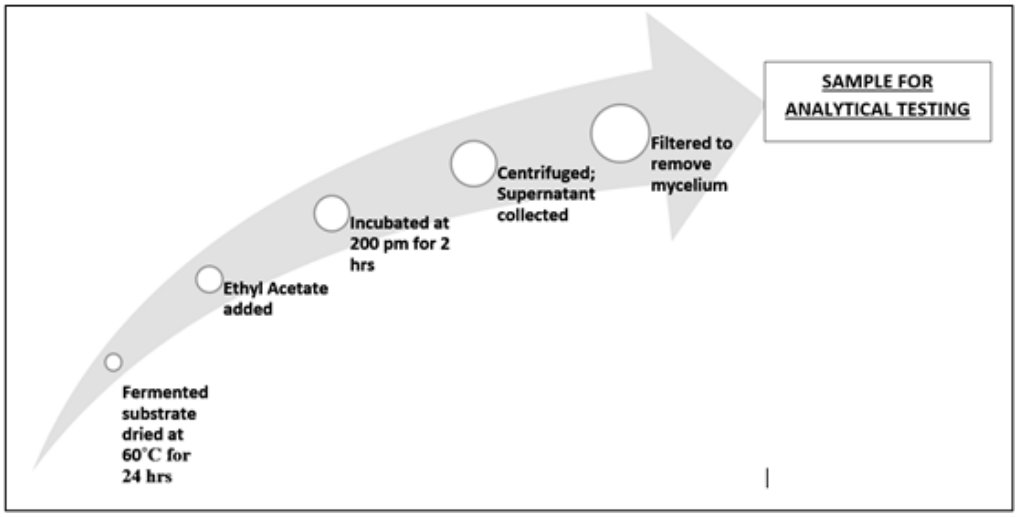


Fig 5: Procedure for extraction of statins

3. Analysis

Once the extracts were obtained, the samples were tested using the TLC method. The TLC plate was initially activated by heating in the oven for 1-2 mins. A minute quantity of the extracts was spotted on the plate using a capillary tube. A chromatogram was developed in a solvent system containing hexane and ethyl acetate following which the plates were observed under UV Lamp and RF values were calculated.
1mL of each filtrate was taken for spectrophotometric analysis. 1mL of 1% Acetic Acid was added to each sample and was left for incubation for 10 mins. It was then diluted 10 times with Methanol. Absorbance was taken in UV-Vis Spectrophotometer at 238nm at Dept. of Biotechnology, RVCE. The standard analysis of Lovastatin showed a peak at 238nm. Hence this wavelength was considered for all readings.
Further characterization is carried out by FTIR Analysis of the dried samples. It was carried out at Inter Disciplinary Research Centre, RVCE, Bangalore. An IR Spectra (found through literature survey) of Standard Lovastatin was used as the reference to compare the spectra which were obtained for the samples.

Three samples namely, Raw Banana, Pomegranate and Potato were analyzed for purity using HPLC. These three samples were chosen as these were the samples which showed the maximum amount of Lovastatin as per our previous studies and analysis methods. The mobile phases used were 100% Acetonitrile and 0.5% Acetic acid in the ratio 70:30. The run time of each individual sample was 15 minutes. According to literature survey, pure Lovastatin is known to be crystalline in nature and has a characteristic Lactone ring which has a peak at the 11th minute. The hydroxyacid form of Lovastatin is found in plant extracts and is a resultant of

solid state fermentation. This can further be purified to obtain pure Lovastatin. The peak for this hydroxyacid was found at 6.6th minute [14][15][16].

The standard image was of crystal morphology of pure drug (Lovastatin) and modified crystals obtained in presence of additives were studied by scanning electron microscopy. SEM of the dried samples were carried out at Inter Disciplinary Research Centre, RVCE, Bangalore. The images were obtained from SU-1500: Scanning Electron Microscope. All the images obtained in SEM analysis were under the same conditions having; Acceleration Voltage: 5000 Volt, Magnification: 5000, Emission Current: 67000 nA. The working distance and length scale varies for every image. The length scale taken for every sample was 10µm, 20µm, 50µm, 100µm and 1mm.

4. Results and discussion

4.1 Yield

Yield of dry waste is calculated as:

$$Yield\ of\ dry\ waste\ = \frac{weight\ of\ dry\ powder\ after\ particle\ size\ analysis}{weight\ of\ fresh\ waste\ initially\ taken} \times 100$$

Table 2: Weights and yield of powdered samples

DRYING AND POWDERING OF WASTE				
	Fresh weight	**Final weight after drying**	**Final powdered weight after Particle Size Analysis**	**Yield`**
Raw Banana	30g	14g	12g	40%
Onion	20g	10g	8g	40%
Potato	47g	28g	28g	59%

Orange	33g	14.2g	14g	42.4%
Lemon	10g	9.2g	9g	90%
Pomegranate	40g	32.64g	32g	80%

From the particle size analysed powdered samples, 5g of each was taken for growth of the *A. terreus spp.* Thus, the yield % has been calculated accordingly as follows:

$$Yield\ of\ dry\ extract\ = \frac{weight\ of\ vacuum\ dried\ sample}{weight\ of\ dried\ waste} \times 100$$

Table 3: Yield of vacuum dried extracts

EXTRACTION OF FOS			
	Initial weight of powdered waste	**Weight of vacuum dried extract**	**Yield**
Raw Banana	5g	1.34g	26.8%
Onion	5g	1.16g	23.2%
Potato	5g	1.2g	24%
Orange	5g	1.1g	22%

Lemon	5g	1.21g	24.2%
Pomegranate	5g	1.19g	23.8%

4.2 TLC

Thin layer chromatography was performed to confirm the presence of statins in the extracts. The total run length for the mobile phase was 7.9 cm. By considering this length, the Rf values were calculated. The TLC plates observed under Long UV are as follows:

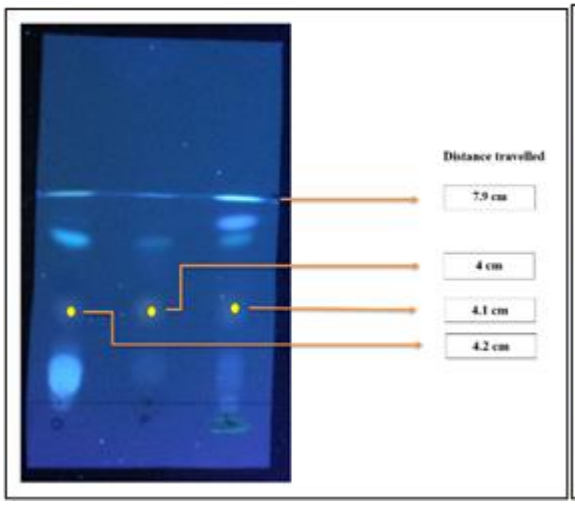


Fig 6: TLC Plate for fruit samples

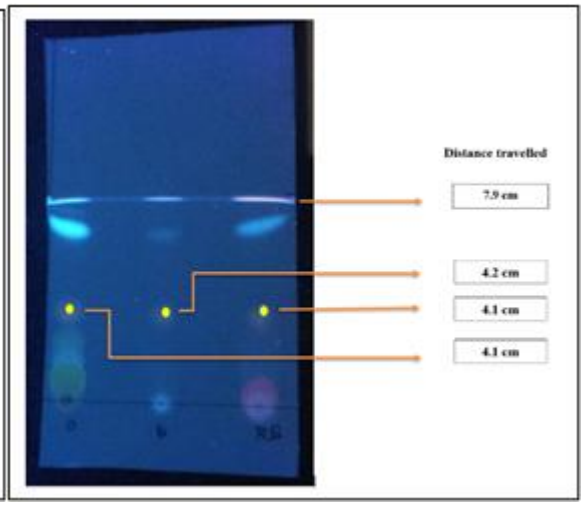


Fig 7: TLC Plate for vegetable samples

Table 4: Rf values of samples

Point	**Distance travelled (in cm)**	**Rf value**
Orange	**4.2**	**0.53**
Pomegranate	**4**	**0.506**

Lemon	**4.1**	**0.51**
Onion	**4.1**	**0.51**
Potato	**4.2**	**0.53**
Raw Banana	**4.1**	**0.51**

From the chromatograms developed, it was observed that the Rf values obtained were in synchronization with that of the standard Lovastatin (i.e. 0.52) [17]. Thus, the presence of Lovastatin is confirmed in all the samples.

4.3 Spectrophotometric Analysis

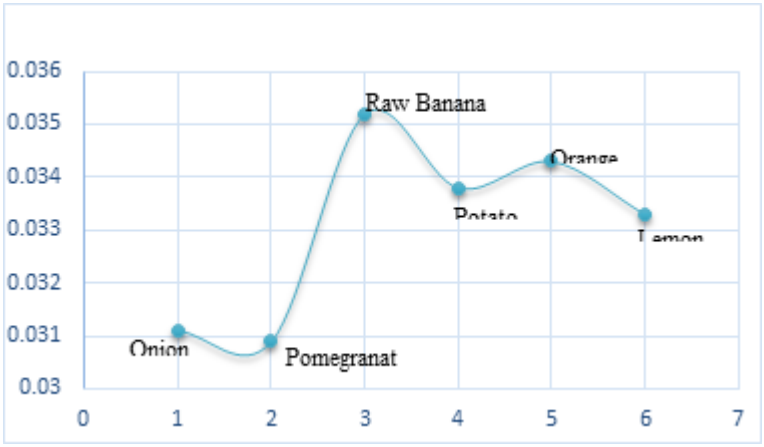
The spectrophotometric analysis was performed at 238nm since Lovastatin was detected at this wavelength. The results for the analysis are shown in as follows:

Table 5: Absorbance at 238nm for different samples and their corresponding concentrations

SAMPLE	**O.D at 238nm**	**CONCENTRATION (mg/ml)**
Onion	2.501	0.0311
Pomegranate	2.484	0.0309
Raw Banana	2.854	0.0352
Orange	2.734	0.0338
Potato	2.774	0.0343

Lemon	2.688	0.0333

From the above results, it was concluded that the raw banana and potato samples showed the maximum concentration of Lovastatin.



Graph 1: Samples and their concentrations

4.4 FTIR

From the graphs so obtained from FTIR analysis, the readings so obtained were compared with the ones of the standard curve [18].

The absorbance curve for vegetable extracts:

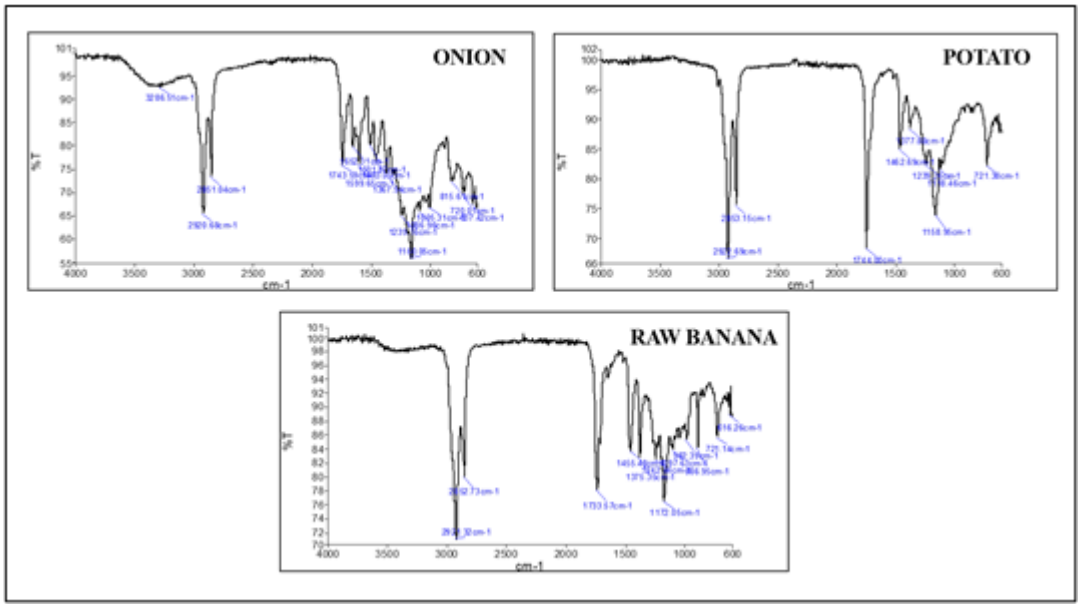


Fig 8: FTIR Spectra of vegetable samples

The absorbance curves for the fruit extracts are as follows:

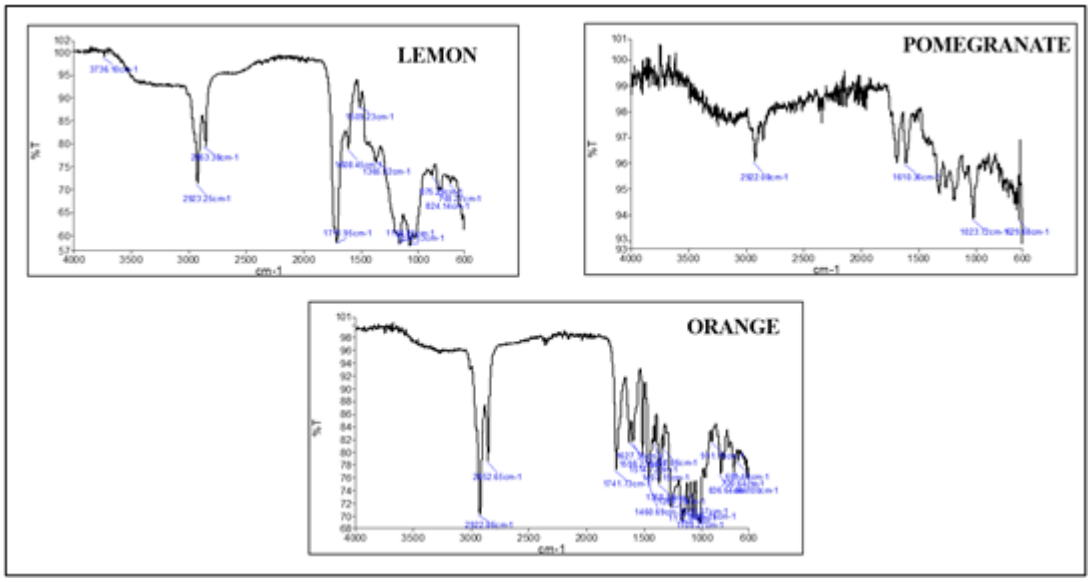


Fig 9: FTIR Spectra of fruit samples

Upon comparison with the IR standard curve of Lovastatin standard, it was found that Raw Banana showed the peaks closest to the ones of the standard. Potato also showed a very good peak graph. Orange and lemon also showed a close similarity with the standard graph.

All the samples taken for study showed the presence of Lovastatin with respect to the peaks obtained. Pomegranate sample showed a slight deviation with respect to peaks within the range of 3000-100 cm -1.

4.5 HPLC

Upon comparing these results with the standard graph, the following conclusions can be brought out:

- For the Raw Banana sample, the peak at 7.331st minute and 11.643rd minute confirmed that the hydroxyacid form and lactone ring form are present. However, the area% for the area under the peak at 7th minute was not satisfactory. Upon repeating of the sample run, a closer peak can be obtained.
- For the Pomegranate sample, the peak at 11.442nd minute is the closest similar peak. The peak for presence of Lactone ring was not present.
- For the Potato sample, both the peaks were obtained at 6.8th and 11.5th minute. This was in synchronization with the peaks of the standard.

Another major peak at 4th minute was observed in all the samples. This is the peak of Ethyl acetate since the samples were prepared in ethyl acetate [20].

Sample Name	Area % for Hydroxyacid peak	Area % for Lactone peak	Total Area%
Raw Banana	0.53%	7.192%	7.722%
Pomegranate	0.021%	0.704%	0.725%
Potato	6.03%	9.025%	15.055%

From literature survey, it was known that the value of Area% is directly proportional to that of the Purity % Thus, from HPLC Analysis, we can confirm that the sample of potato extract has shown the maximum purity as compared with that of the others [21][22].

4.6 Scanning Electron microscope

The SEM showed that the Lovastatin extracted from each sample was having irregular size and shape of particles. Onion and Potato have shown very similar images to that of the standard image. The extract from Pomegranate has shown fibre like structure owing the fibrous nature the fruit peel.

However, the extract from lemon, raw banana and orange did not dry completely inspite of days of drying. This could be because of the presence of pectin which is found in high amounts in all fruit peels.

Therefore, the images from these show a paste-like image of the extracts. This may also correspond to the nature of composition of these peels. As this is not a highly purified form rather just a crude extract of lovastatin, it may be containing other compounds which explains the paste-like images.

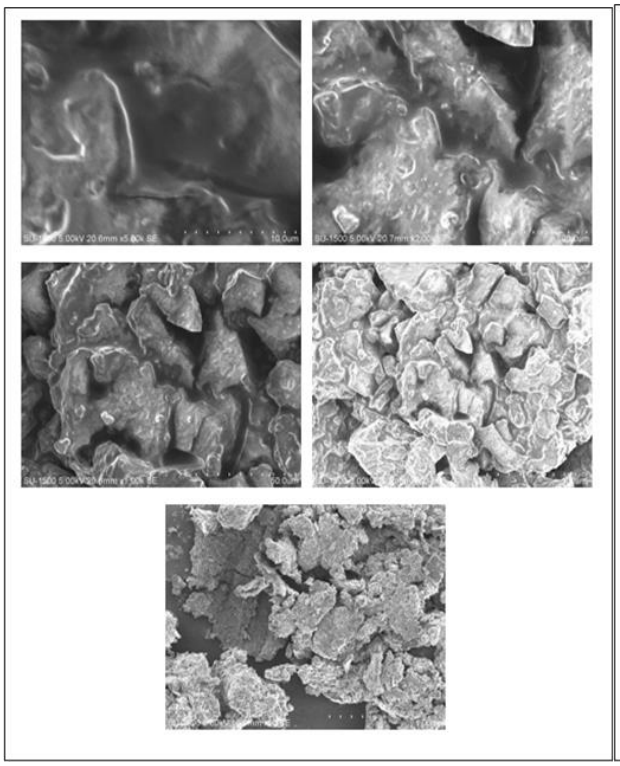


Fig 29: SEM of Onion extract

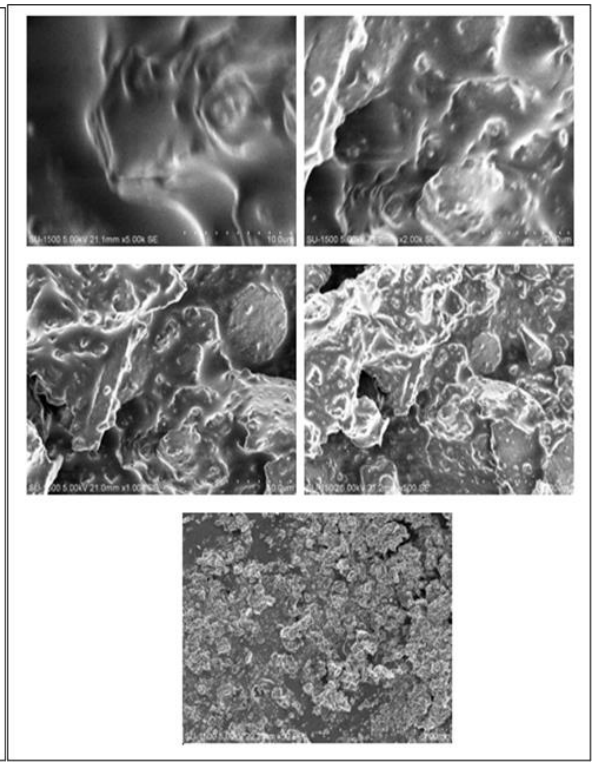


Fig 30: SEM of Potato extract

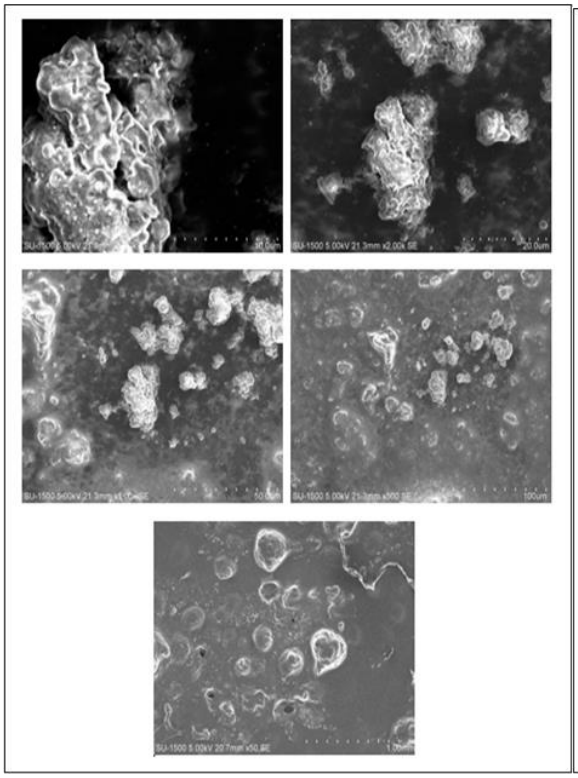


Fig 31: SEM of Raw banana extract

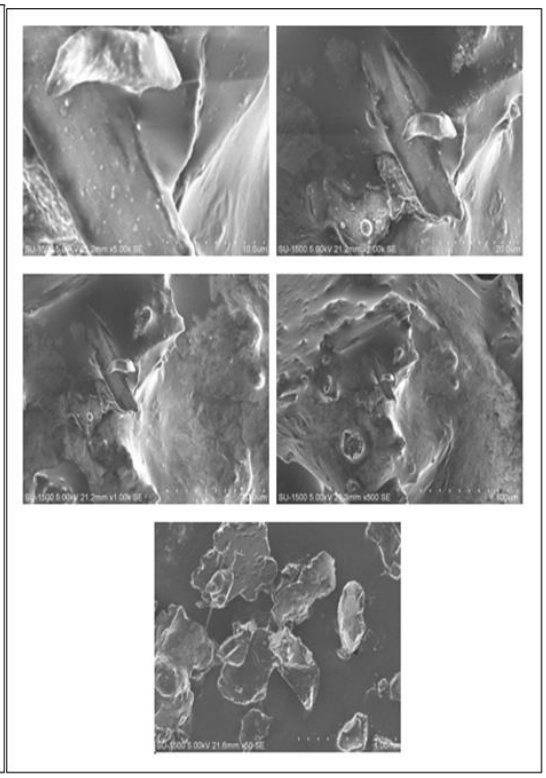


Fig 32: SEM of Orange extract

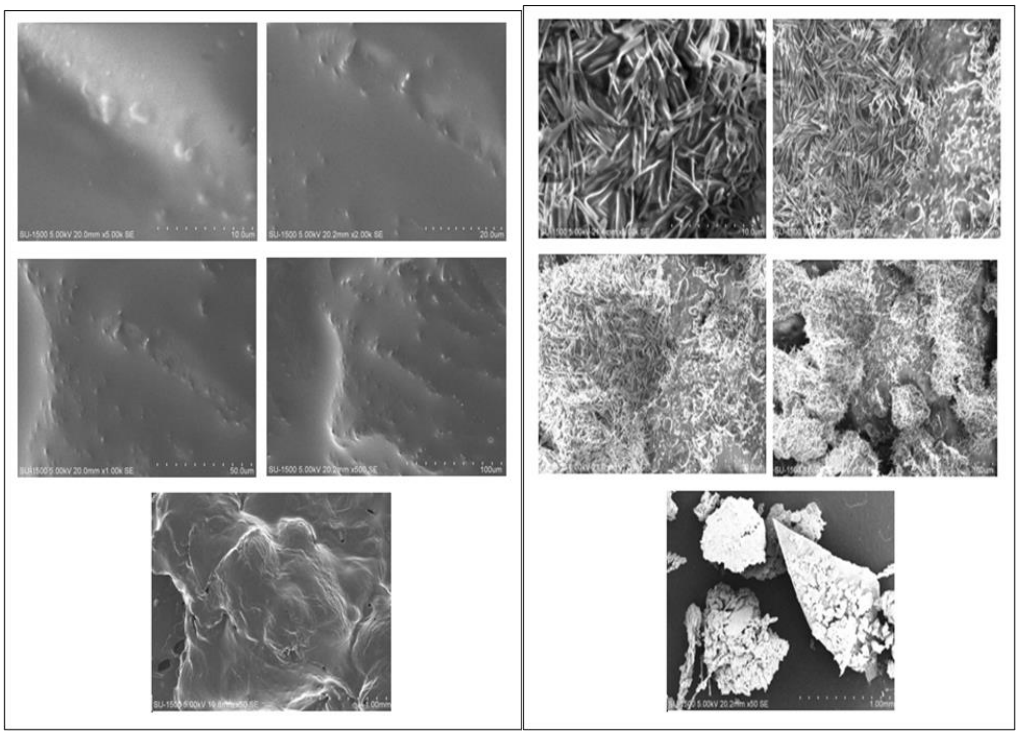


Fig 33. SEM of Lemon extract **Fig 34.** SEM of Pomegranate extract

5. Conclusion

Lovastatin is a statin drug used to lower cholesterol and thereby reduce the risk of cancer. Organic waste substrates like vegetable peels-onion, potato and raw banana and fruit peels-lemon, orange, pomegranate was used as substrates to carry out solid state fermentation and lovastatin was extracted using ethyl acetate. The presence of lovastatin extracts was confirmed by the TLC, Spectrophotometric analysis and HPLC. The study proceeded in a positive direction yielding the expected results. Three samples were considered for analysis, Raw banana, pomegranate, potato. The rf values obtained from TLC for a total run length of mobile phase of 7.9cm, corresponded with that of standard lovastatin (0.52), confirming the presence of lovastatin in all samples. It was concluded that raw banana and potato samples showed maximum lovastatin concentrations by spectroscopic analysis carried out at a wavelength of 238nm, optimum for lovastatin detection. Further, HPLC studies were carried out on the raw banana and potato samples to analyze the purity of the obtained lovastatin. It was observed that for the Potato sample (FIG 5.), both peaks obtained at 6.8 th and 11.5 th minute were in synchronization with the peaks of the standard. However, it was noticed that the area under the peaks for the samples is very less as compared to the >98% of the pure Lovastatin due to the samples being in the crude form. A permanent and maximized area percentage can be obtained by thorough purification of the sample. Although HPLC confirmed the presence of lovastatin in all three samples, the potato sample was found to be most pure. Further, economic cost comparison analysis was performed which proved that this can serve as a cost-effective economic solution to waste management and lovastatin extraction.

Optimization of conditions like pH and temp have been carried out in these studies, facilitating the yield of positive results allowing us to definitively conclude that the organic waste materials used above, potato peels in particular, are good sources of Lovastatin [24]. Thus, utilizing this extraction process will pave the way for economical and eco-friendly methods for production of useful substances like Lovastatin from organic wastes of fruits and vegetables. Furthermore, fruits and vegetables are a good source of antioxidants with the highest capacity found in the outer sections. Therefore, this waste could be used to produce functional ingredients with important health benefiting properties, due to the presence of bioactive compounds.

This study serves as an illustration of the innovative solutions that can be devised to handle organic waste efficiently contributing to domestic waste reduction. Future scope for this study includes purification, optimization and economic cost comparison analysis for this extraction process.

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