



## Research Article

### ANTI-CANCER POTENCY AND SUSTAINED RELEASE OF PHYTOSOMAL DIALLYL DISULFIDE CONTAINING METHANOLIC *ALLIUM SATIVUM* EXTRACT AGAINST BREAST CANCER

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#### ABSTRACT

Vesicular drug delivery system like phytosomes is a widely adopted section of pharmaceutical inventions. It improves therapeutic efficacies of drug by controlled and sustained action. It is also used to improve the therapeutic index, solubility, stability and rapid degradation of drug molecules. Phytosome is made of phytoconstituents of herbal extract that is surrounded and bounded by one or more concentric spheres of lipid layers. The purpose of this study is to synthesize an economical phytosome which will also be an effective alternative to the current medications of cancer. Methanolic extract of *Allium sativum* containing Diallyl disulphide along with other phenolic compounds were used for the preparation of phytosome as it has the ability to cure and prevent the growth and division of cancer cells. Bioactive compounds were examined by phytochemical analysis. Antioxidant activity of the extract was carried out by DPPH assay that showed that the extract was rich in antioxidants. Presence of Diallyl disulfide having the anti-cancer activity was confirmed by HPLC and GC-MS analysis. The surface morphology and the functional groups of the prepared phytosomal complex were studied by SEM and FTIR analysis respectively. The prepared phytosome showed 100% toxicity against the cancer cell line (MCF 7) at 108.5 µg/ml. Hence, we claim that the Diallyl disulphide containing methanolic *Allium sativum* encapsulated in phytosome can be an effective alternate for the cancer therapies and this work can also be extrapolated to active targeting of tumour site by attaching the targeting moiety on the surface of the phytosome.

**Keywords:** Vesicular drug delivery system, Phytosome, Diallyl disulfide, methanolic extract, *Allium sativum*, Breast Cancer, MCF-7.

#### INTRODUCTION

Drug delivery refers to approaches, formulations, technologies, and systems for conveying a drug compound in the body as needed to safely accomplish its desired therapeutic effect<sup>1</sup>. Conventional drug delivery system is not effective due to limited permeation of drugs into cells. Therefore, considerable attention has been focused on the development of novel drug delivery system. In recent years, vesicles as a carrier system have become the vehicle of choice for drug delivery due to their enhanced efficiency, cost effectiveness and also an alternative targeting method for drug delivery at the site of infection by improved bioavailability<sup>2,3</sup>. This system delays drug elimination of rapidly metabolizable drugs, and function as sustained release systems<sup>4</sup>.

Phytosome is a complex of a natural active ingredient and a phospholipid. It is an emerging technique applied to phyto-pharmaceutical which contains phytoconstituents of herbal extract surrounded and bounded by lipid bilayer<sup>5,6,7</sup>. It protects valuable component of herbal extract from destruction by digestive secretion and gut bacteria<sup>8,9</sup>. This shows better absorption of the drug which produces better bioavailability<sup>10</sup> and improved pharmacological and pharmacokinetic parameters than conventional herbal extract<sup>11,12</sup>.

There are few reports on the anticancer activity of Diallyl disulfide<sup>13,14,15</sup> but this is the first report where a novel complex is prepared by reacting equimole of natural phospholipid and

methanolic extract of *Allium sativum* which has Diallyl disulfide exhibiting 100% mortality against cancer cells (MCF-7) and sustained drug release profile.

#### MATERIALS AND METHODS

##### Preparation of plant extract

*Allium sativum* was bought from local market, Erode, Tamil Nadu, India. Cloves of *Allium sativum* were mashed with mortar and pestle. Methanolic extract was prepared using Soxhlet apparatus at 65°C. Obtained extract was stored at 20°C and used when required<sup>16</sup>.

##### Phytochemical analysis

The methanolic extract of *Allium sativum* is analysed for its phytoconstituents such as saponin, phenol, tannin, flavonoid, terpenoids and phlobatannin<sup>17</sup>.

##### HPLC analysis

The chromatographic detection of Diallyl disulfide was performed using a Zorbax SB - C<sub>18</sub> column (internal diameter, 2mm; length 150mm; particle size 3µl) and a guard column (Phenomenex, Torrance, CA). The solvent system used was a gradient of n-hexane and 2-propanol in 3:1 ratio. The flow rate was set up at 0.7 ml/ min L. Injection volume was 10 µL. Detection was accomplished with photodiode array detector at 240 nm<sup>18</sup>.

### GC-MS analysis

GC-MS analysis of methanolic extract of *Allium sativum* was analysed using Thermo GC - Trace ultra ver: 5.0, Thermo MS DSQ II gas chromatography equipped with DB 35 - MS capillary standard non - polar column dimension of 30mts and mass detector (EM with replaceable horn) was operated in EMV mode. Helium was used as carrier gas with the flow rate of 1.0 ml min<sup>-1</sup>. The injection port temperature was operated at 260°C. The column oven temperature was held at 70°C for 2min then raised to 260°C at 6°C min<sup>-1</sup>.

### Synthesis of phytosome

Phytosome was prepared by complexing phospholipid with the methanolic extract of *Allium sativum* in 1:1 ratio with continuous stirring by solvent evaporation technique. Then the complex was allowed to dry in hot air oven at 65°C till the organic solvent is removed. The resulting phytosomal suspension was stored at room temperature<sup>19</sup>.

### Characterization of phytosome Scanning Electron Microscopy

The surface morphology of the phytosome was studied by recording their SEM micrographs using scanning electron microscope (JEOL-MODLE 6390) at 20KV for the magnification range of 7500.

### FTIR analysis

For FT-IR analysis, prepared phytosome was recorded on Shimadzu FT-IR 8400S and MB3000 in KBr matrix over a spectral width of 4000 to 500cm<sup>-1</sup> at a resolution of 2cm<sup>-1</sup>.

### Entrapment efficiency

Phytosome was centrifuged at 15000rpm for 45 min to separate phytosomes from free drug. Concentration of the free drug as the supernatant was determined by measuring absorbance at 217nm using UV spectrophotometer. The percentage drug entrapment was calculated by using the formula<sup>20</sup>,

$$\text{Entrapment efficiency} = \frac{\text{Wt of total drug} - \text{Wt of free drug}}{\text{Wt of total drug}} \times 100$$

### In vitro drug release study

A 1 gm scoop of the synthesized phytosome was added in to a beaker containing 150ml of Phosphate Buffer Saline at pH 7.4. Solution was stirred at 300 rpm using magnetic stirrer at 37°C. At a regular time intervals of hours, 3ml of the solution was withdrawn from the stirring solution and immediately compensated with fresh Phosphate Buffer Saline (pH 7.4). The samples were analyzed spectrophotometrically at 217nm and the percentage drug release was determined<sup>20,21</sup>.

### Release kinetics

Release kinetics of the DADS containing *Allium sativum* extract from phytosome was studied using Korsmeyer-Peppas equation where the linear regression of Log(M<sub>t</sub>/M) Vs Log(t) curve indicates the release model of the phytosome<sup>22</sup>. To elucidate the mechanism of drug release, first 60% drug release data was fitted in Korsmeyer-Peppas model,

$$F = (M_t/M) = K_m t^n$$

Where F is fraction of drug released at time 't', M<sub>t</sub> is amount of drug released at time t, M is total amount of drug in dosage form, K<sub>m</sub> is kinetic constant, n is the diffusion or release exponent and t is time in hours.

'n' is estimated from linear regression of log (M<sub>t</sub>/M) Vs log (t)

### Antioxidant activity

The antioxidant activity of the plant extract was examined on the basis of the scavenging effect on the stable DPPH free radical activity by where the working solution was prepared at different concentrations (0.5, 1, 1.5, 2 and 2.5 mg/ml)<sup>23</sup>. The percentage inhibition of DPPH activity was calculated using the formula

$$\% \text{ inhibition of DPPH activity} = \frac{(A_c - A_s)}{A_c} \times 100$$

Where, A<sub>c</sub> = Absorbance of control

A<sub>s</sub> = Absorbance of test sample

### In vitro cytotoxicity assay

In vitro cytotoxic activity of the phytosome was tested by Methylthiazol Tatrazolium assay. The percentage viability was calculated using the following formula<sup>24</sup>.

$$\% \text{ Viability} = \frac{\text{Mean OD of individual test group} \times 100}{\text{Mean OD of control group}}$$

## RESULTS AND DISCUSSION

### Phytochemical analysis

Phytochemical investigation was carried out to determine the active components in the methanolic extract of *Allium sativum*. Based on the calorimetric reactions, the possibility of isolable compounds in the extracts of *Allium sativum* are tabulated (Table 1). This shows that the extract offer a wider array of phytochemicals.

Some reports stated the presence of similar compounds in phytochemical analysis of the methanolic extract of *Allium sativum*<sup>25,26</sup>.

**Table 1: Phytochemical analysis of methanolic extract of *Allium sativum***

Phytoconstituents	Methanolic extract of <i>Allium sativum</i>
Saponin	+
Phenol	+
Tannin	+
Flavonoid	+
Terpenoid	+
Phlobatannin	-

(+) Present (-) Absent

### HPLC analysis

The peaks obtained from methanolic extract of *Allium sativum* analyzed by HPLC analysis is shown in the Figure 2. The peak at 5.4 minutes RT while eluting the crude extract proved the presence of Diallyl disulphide on comparing with the standard Diallyl disulfide elution peak (Figure 1). The determination of Diallyl disulfide by HPLC analysis from macerated garlic oil at the same retention time with similar ratio of solvents as in this finding were reported<sup>18</sup>.

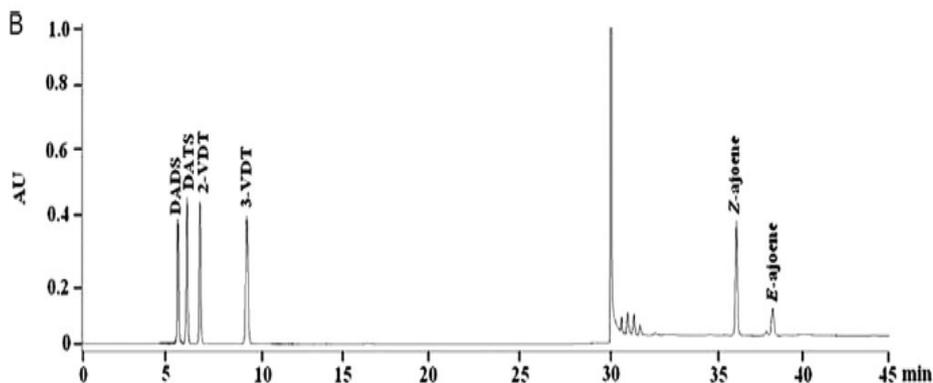


Figure 1: HPLC chromatogram of standard DADS<sup>18</sup>

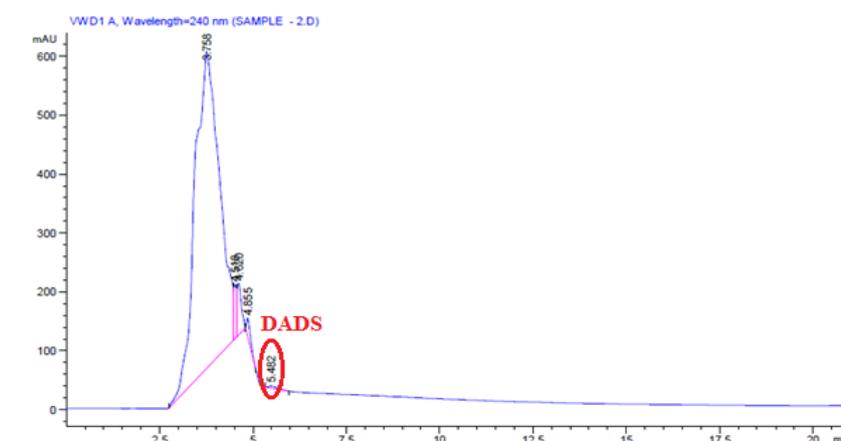


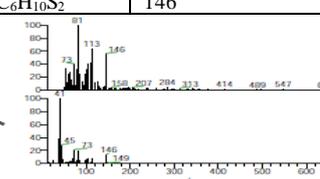
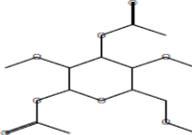
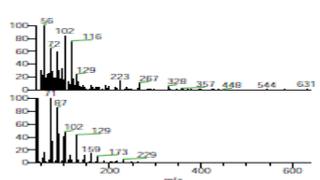
Figure 2: HPLC chromatogram of methanolic extract of *Allium sativum*

**GCMS analysis**

The GC-MS analysis of methanolic extract of *Allium sativum* revealed the presence of phytochemical constituents that contributes to anticancer activity and phytosome formation such as Diallyl disulfide and α-D-Mannopyranose, 2,4,6-tri-O-methyl-diacetate respectively are tabulated (Table 2).

Diallyl disulfide is identified as significant component in crude methanolic extract of *Allium sativum* which can perform anti-cancer effects<sup>27</sup>. The comparison of *Allium sativum* from Bangladesh and China by GCMS and the presence of Diallyl disulfide in both were reported<sup>28</sup>.

Table 2: Phytochemicals identified in the methanolic extract of *Allium sativum* by GCMS analysis

S.No	Retention time	Compound	Molecular formula	Molecular Weight	Area %
1	6.78	Diallyl disulfide	C <sub>6</sub> H <sub>10</sub> S <sub>2</sub>	146	2.86
Structure	<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>Diallyl disulfide Formula C6H10S2, MW 146, CAS# 2179-57-9, Entry# 1979 Allyl disulfide</p>  </div> <div style="width: 50%;">  <p>NL: 9.99E2 GCMS: 33185_6.78 1 6.00 5 8.00E-4 + S Full ms [50.00-650.00]</p> <p>NL: 9.99E2 SI 554_1251_650 massib, Entry# 1979, CAS# 2179-57-9, Diallyl disulfide</p> </div> </div>				
2	4.86	α-D-Mannopyranose, 2,4,6-tri-O-methyl-, diacetate	C <sub>13</sub> H <sub>22</sub> O <sub>8</sub>	306	0.38
Structure	<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>α-D-Mannopyranose, 2,4,6-tri-O-methyl-, diacetate Formula C13H22O8, MW 306, CAS# 55255-91-7, Entry# 6520 1,3-O-(1-acetyl-2,4,6-tri-O-methylheosopyranose)-β</p>  </div> <div style="width: 50%;">  <p>NL: 9.99E2 GCMS: 3391_4.86_1 6.00 5 3.61E-4 + S Full ms [50.00-650.00]</p> <p>NL: 9.99E2 SI 432_1251_658_main1b 55255-91-7, CAS# α-D-Mannopyranose, 2,4,6-tri-O-methyl-, diacetate</p> </div> </div>				

### Synthesis of phytosome

The phytosome was prepared by complexing phospholipid and methanolic extract of *Allium sativum* in 1:1 ratio and were dried is shown in the Figure 3.

The most preferable ratio of phospholipid to plant component for phytosome formation is 1:1 were reported<sup>29</sup>. Silybin-phospholipid complex were prepared using ethanol as a reaction medium<sup>30</sup>. Silybin and phospholipids were resolved into the medium, after the organic solvent was removed under vacuum condition, silybinphospholipid complex was formed. There are few reports that described the methods of phytosome preparation<sup>31,32,33</sup>.



Figure 3: Photograph of the as prepared Phytosome after dried.

### Characterization of phytosome SEM analysis

From the SEM image, the morphology of the phytosomes was determined to be spherical in shape. The polar heads on the phytosomic particles were observed in the Figure 4. At 30,000x magnification, the size of the phytosome was found to be 500nm. The size of mitomycin – soybean phosphatidylcholine complex loaded in phytosome was found to be 500nm<sup>34</sup>.

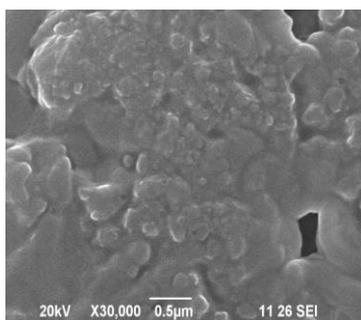


Figure 4: SEM image of Phytosome where 2 dimensional spherical particles can be spotted at submicron scale

### FTIR analysis

The FTIR peak of phytosome and its corresponding functional groups are shown in the Figure 5 and Table 3 respectively. From the FT-IR spectroscopy, the presence of aromatic and lipid functional groups that contribute to the formation of phytosome were identified. The presence of aromatic groups were confirmed by the absorbance peak at 1458 cm<sup>-1</sup> (C=C), 1551 cm<sup>-1</sup> (N-H), 1643 cm<sup>-1</sup> (C=N) and 1744 cm<sup>-1</sup> (C=O) whereas the bands such as 2924 cm<sup>-1</sup> and 2854 cm<sup>-1</sup> mainly represents C-H stretching vibrations that are caused by lipids consolidating the confirmation of phytosome formation.

The phytosomal complex of ethanolic extract and phosphatidylcholine have N-H functional group and C-H stretching vibrations at 2924 cm<sup>-1</sup> and 2854 cm<sup>-1</sup>.<sup>135</sup>The occurrence of C-H stretching vibrations caused by lipids and N-H, C=O, C=C functional groups consolidate the formation of phytosome<sup>19</sup>.

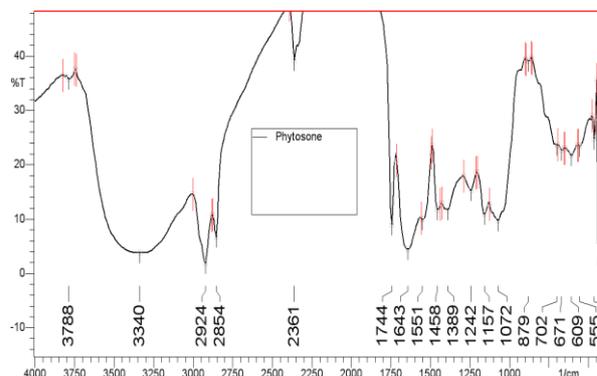


Figure 5: FT-IR peak of Phytosome revealing the functional groups

Table 3: Wave number (cm<sup>-1</sup>) of dominant peak obtained from absorption spectra of Phytosome

S.No	Wave number	Functional group
1	3788	O-H (alcohol)
2	3340	O-H stretching
3	2924	sp <sup>2</sup> C-H stretch and carboxylic O-H stretch
4	2854	C-H (sp <sup>3</sup> carbon)
5	2361	Thiol S-H stretch
6	1744	Unconjugated carbonyls
7	1643	C=O aromatic conjugated; C=N bond; C=C bond
8	1551	C=C stretch aromatic; N-H bend; Nitro
9	1458	C=C stretch aromatic
10	1389	C-N stretching
11	1242	C-O stretching
12	1157	C-O
13	1072	C-O deformation
14	879	C-H out of plane deformation
15	702	phenyl ring out of plane ring deformation
16	671	Phenyl group
17	609	Bending O-C-O
18	555	Deformation of C-C-O and C-C-C

### Entrapment efficiency

The entrapment efficiency of prepared phytosomal complex of phospholipid and *Allium sativum* extract was determined to be 97.306 %. The entrapment efficiency of different formulation of phytosomes were higher than 90% due to high liposolubility of garlic oil<sup>36</sup>. The entrapment efficiency of liposome that contain *Allium sativum* extract was 47.5±7.3% which shows that the phytosome has a property of higher entrapment efficiency than liposome<sup>37</sup>.

### In vitro drug release study

The comparison of percentage drug release of DADS encapsulated *Allium sativum* phytosome and methanolic extract of *Allium sativum* in Phosphate Buffer Saline is shown in the Figure 6. The phytosome showed better percentage release of drug than the methanolic extract of *Allium sativum*. The percentage release of extract from phytosome at different hours

was depicted in the graph. At 0<sup>th</sup> hour, there was 82.31% of release of drug which accounts for desorption of extracts from the hydrophilic surface of the phytosome along with the extract released from the core region of the phytosome. Further release for every 30 minutes were controlled with respect to time and at 3<sup>rd</sup> hour there was 85.35% release.

The capsules of Ashwagandha phytosomes (76.8%) has highest cumulative % drug release compared to conventional formulation (50.2%)<sup>38</sup>. The percentage release of garlic oil from the garlic oil - SLN complex in the medium and was found to be that the drug release was continuous and slow, indicating the drug release rate was determined by the diffusion of the drug from the rigid matrix structure<sup>36</sup>.

But both these reports were time independent and concentration dependant. In our report, the release was time dependant making the release kinetics, reliable for treatment and dosage delivery.

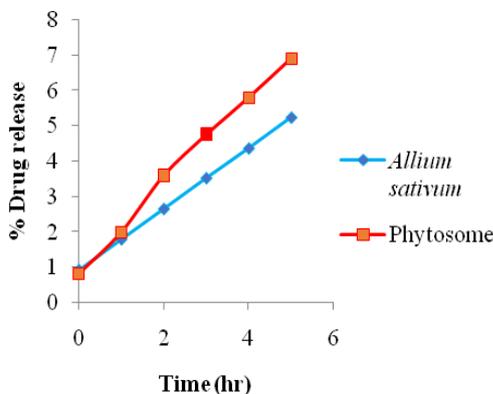


Figure 6: Percentage drug release of Phytosome and Allium sativum

**Release kinetics**

Value of ‘n’ 0.929 indicates that the release was zero order, non-fickian diffusion of extract from the phytosome. It is independent of concentration and time dependent. This makes it ideal drug delivery mode since the release is not affected by concentration gradient over a period of time. The release kinetics of plant extract Phyllanthin from phytosome was evaluated by Sandeep et al., and found the value of n to be in the range of 0.912 to 0.998 for their different formulation<sup>39</sup>. This correlation reveals the release efficiency of phytosome that the vesicle is suitable for sustained release of polyphenolic plant extracts.

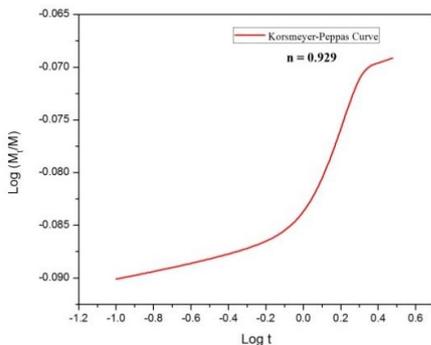


Figure 7: KorsmeyerPeppas curve of Log (Mt/M) Vs Log t where linear regression n was found to be 0.929

**Antioxidant activity**

The results of antioxidant activity by DPPH assay shows that the percentage inhibition increases with increase in concentration of the methanolic extract as indicated in Figure 8 and the IC 50 obtained is 0.42 mg/ml.

The Allium sativum was reported to have high in antioxidants<sup>40</sup>. Organosulfur compounds of Allium sativum such as allin and diallyl disulphide were reported to have antioxidant as well as anticancer properties<sup>41</sup>. Antioxidant potency of the phytosome depends on the release of extract. Extract released from the phytosome has no physicochemical difference from the unloaded DADS containing Allium sativum extract. Hence antioxidant activity of phytosome with respect to time has been depicted in the Figure 9.

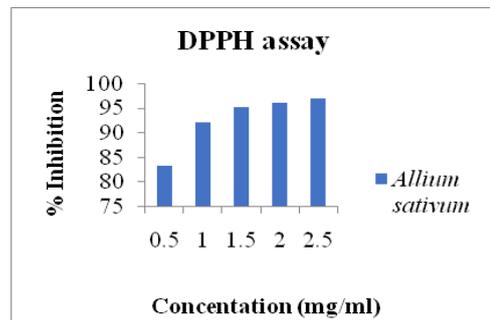


Figure 8: DPPH assay of methanolic extract of Allium sativum in which at the minimal concentration of 0.5 mg/ml, there was 83.2% inhibition of free radicals is observed

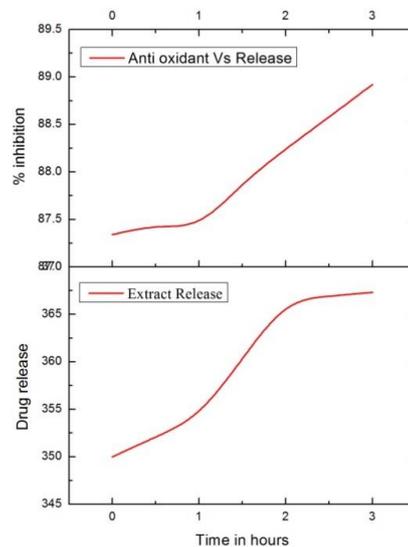


Figure 9: Antioxidant activity based on release of extract from the phytosome

**In vitro cytotoxicity assay**

In vitro evaluation of cytotoxic activity was evaluated by MTT assay. The percentage cell viability of breast cancer cells treated with phytosome at different concentrations is displayed in Figure 10. and showed IC<sub>90</sub> was achieved at 108.5 µg/ml. The typical microscopic image of the cell before and after treating with phytosome were shown in the Figure 11 which indicates that the prepared phytosome is 100% toxic to breast cancer cell lines.

The FA-PEG-MMC loaded phytosomes showed better cytotoxic effect and anticancer activity compared to the free MMC injection<sup>34</sup>. Silymarin phytosome show better antihepatotoxic activity than silymarin alone in broiler chicks that protect against the toxic effects of aflatoxin B1<sup>42</sup>. The mechanism behind improved cytotoxicity remains unstudied.

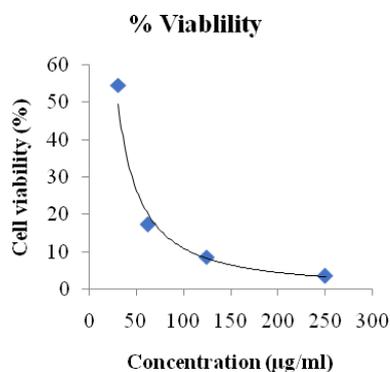


Figure 10: Variation of percentage cell mortality at different concentrations

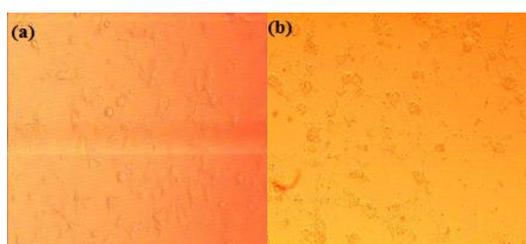


Figure 11: Microscopic image of breast cancer cell line (a) Cell control (b) After treatment

## CONCLUSION

Phytosome prepared through interaction between phospholipid and methanolic extract of *Allium sativum* that contains DADS has been observed to be effective scavenger of reactive free radicals and showed strong antioxidant activity. This property which is also responsible for anti-cancer activity has been confirmed and the  $IC_{50}$  value was identified to be 25.76 µg/ml and  $IC_{90}$  was 108.5 µg/ml. DADS responsible for the antioxidant and anti-cancer activity was confirmed to be present in the extract. Percentage release of DADS from phytosome illustrates the sustained release of drug over time. The release was found to be time dependent and independent of concentration which ensures the controlled dosage release of extract. This proves that the DADS containing *Allium sativum* methanolic extract encapsulated in phytosome can be better mode of drug delivery with enhanced efficacy for breast cancer therapy. This study reveals that phytosomes exhibits better pharmacokinetic profile than conventional herbal extract. Many areas are to be explored in near future in the with respect to pharmaceutical applications.

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