ABSTRACT
Cancer is most dreadful leading disease in world, where as from all cancer, lung cancer possesses high mortality rate. Operatively removal of is difficult one and patient is totally depends on chemotherapeutic agent. Hence forth there is need to prepare such formulation having potent anticancer activity and give extended release which minimizes the dosing frequency. Andrographolide a new potent phytoceutical was selected which act on multiple stages of cancer. Here chitosan based folate anchored microparticles of andrographolide were prepared by spray drying method. Conjugation of chitosan and folic acid was prepared this approach was selected as folate receptors are over expressed in cancer condition. Microparticles were prepared in (2:1, 1:1, 1:2, 1:3, and 1:4) proportion with addition of (Dry Powder Inhaleur) DPI carrier- lactose. All DPI batches were analyzed for in vitro dissolution study and in vitro deposition study. Attempt was made to prepare corrugated particles by optimizing spray drying parameters which was obtained successfully at inlet temp 120°C. The optimized batch were further subjected to studies like FTIR, SEM, XRPD and DSC. Additional parameters such as biological activity -angiogenesis, cell line study was carried out to check anti-cancer potential of andrographolide and optimized batch. Hence DPI form of andrographolide microparticles can be used as a successful targeted anticancer for lung tumour.

Keywords: Rographolides microparticles, Lung tumour.

INTRODUCTION
Lung cancer is the most dreadful and high mortality rate cancer amongst all cancers. Especially in United States, mortality rate of lung cancer is many folds more than colon, rectal, breast, and prostate cancer. Till the date, chemotherapy & radiation alone or in combination of both being best line of treatment. Still, both the therapy limits their use in effective treatment of cancer. Radiation therapy does not affect cancer cells throughout the body and chemotherapy uses toxic chemicals that affect healthy as well as cancerous cells. Ultimately, it leads to detrimental side effects like loss of hair, skin problems, mouth sores, and fatigue. Thus need has been felt about effective cancer therapy which can treat the diseased condition without harming the host. Nowadays, Plant-derived compounds are becoming great interest in cancer treatment due to their fewer side effects. Campothecin derivatives, Topotecan and Irinotecan, Etosopside, derived from Eppipodophyllotoxin and Paclitaxel from taxol Furthermore, other potent molecules include Vinca alkolloids (Vinblastine, Vincristine) etc. Presently, research on anticancer drug development is largely dependent on exploring potential phytochemicals. Numerous new plants coming in to the focus such as curcuma longa, piper longum (Koul, IB et al; 1995), Andrographis Paniculata etc. These above herbs scientifically proven anti-cancerous properties and are used for the treatment of various cancers they work by multiple biochemical pathways and are capable of influencing several organ systems simultaneously.

Thus, in this aspect phytoceutical drug targeting is the best approach to direct a therapeutic agent specifically to the desired site of action with little or no interaction with non target tissue or normal cell of the body. Targetting can be achieved by imparting the tumors nutritional support like transferrin, Hyaluronic acid, Folate etc. Folic acid is a vitamin required essential for the biosynthesis of nucleotide bases and also in several metabolic pathways. Folate receptor is frequently over expressed on cancer cells, perhaps enabling the malignant cell to compete successfully for the vitamin. The attractiveness of the folate receptor has been enhanced by its high binding affinity, low immunogenicity and ease of modification. The receptor of folic acid constitutes a useful target for tumour specific drug delivery folate receptors are up regulated in many human cancers. Folate receptor density appears to increase as the stage /grade of the cancer worsens. Thus, cancers that are most difficult to treat by classical methods may be more easily targeted with folate-linked therapeutics.

Andrographolides microparticles, Lung tumour.

MATERIAL AND METHODS
Chitosan was purchased from Marine Chemicals Pvt. Ltd., Cochin. We also thankful to Research Organic, Chennai, India, for kind gift of andrographolides. N-(3-Dimethyl aminopropyl)-ethylcarbodiimide hydrochloride (EDC) was gift sample from Globex Chemie Pune. Folic acid was a kind gift from Yash Chemicals, Pune. Lung cancer cell lines H522 obtained from National Centre for Cell Science (Pune, India).

Formulation of Folate anchored chitosan microparticles
Folic acid (FA) was dissolved in DMSO (Dimethylsulphoxide) in dark condition as folic acid is sensitive towards light. Stirred the solution till gets dissolved. The surface carboxyl groups of folic acid were activated by addition of EDC with constant stirring for 1 hr. FA- Chitosan solution was prepared by drop wise addition of chitosan solution prepared in acetate buffer. Mixing should be continued for 15 hr in dark condition. The andrographolide solution in methanol was prepared and added slowly to FA-Chitosan solution stirred for 4–5 hr till methanol evaporated. The FA-Chitosan and drug and rographolide were taken in different ratio (2:1, 1:1, 1:2, 1:3, and 1:4). Lactose was added in the final solution prior to spray drying in 1:1 proportion. The prepared spray dried microparticles containing andrographolide has been subjected to further evaluation studies.

In order to attain the tumour targeting, conjugate of folic acid and chitosan has been prepared. This synthesis has been started from the conjugation of folic acid to chitosan which was accomplished by coupling the carboxylic groups of folic acid to the NH₂ in which EDC catalyzed the bond formation. This reaction has been illustrated in Fig. 1.
**MATERIALS AND METHODS**

**Drug Content**

Drug content was determined by accurately weighing 20 mg of microparticles and dissolved in methanol. Solutions then sonicate, filtered by whatman filter paper no. 45 and filtrate was assayed by UV-visible spectrophotometer at $\lambda_{max}$ 223. Drug content was calculated by comparing the absorbance with standard curve.11

**In Vitro Dissolution Study**

Different proportions of andrographolide-chitosan microparticles were taken and dissolution study was carried out to check the release profile of microparticles. Dissolution study was carried out in United States Pharmacopoeia (USP) Type-I (basket) dissolution test apparatus (Electrolab, TDT 08L, Mumbai, India). The dissolution medium was 900 ml of phosphate buffer (pH 7.4) maintained at 37.5±0.5°C containing 1% sodium lauryl sulphate (SLS) at 100 rpm. Aliquots, 5 ml were withdrawn at the interval of 30 min and samples were analyzed spectrophotometrically at 223 $\lambda_{max}$. The same amount of fresh phosphate buffer was used to replace the amount withdrawn from the dissolution media. Analysis of data was done using PCP-Disso V3 software (BVCP, Pune, India).

**In Vitro Deposition**

All the batches of spray dried microparticles were characterised for in vitro deposition by twin impinger to determine respirable fraction. The study has been carried out by manually loading weighed quantity (20±1 mg) of microparticles into size 3 hard gelatin HPMC stick free capsule (Associated capsules Pvt. Ltd., India.) The aerosol performance of the formulations was tested by an impactor-based apparatus twin impinger [Copley Instruments (Nottingham) Ltd]. Rotahaler was used to load microparticles in twin impinger which contained 7 and 30 ml of methanol as a collecting solvent in stage 1 and 2 respectively.

The drug content of all formulations recovered from the twin impinger apparatus was determined by UV spectroscopy. Absorbance was measured at of 223 nm for analysis. Concentration was determined by reference to calibration curve prepared from dilution of stock solution of formulation. Following formulae were used for to calculate fine particle fraction, effective index and emitted fraction.12

\[
\text{Fine particle fraction} = \frac{\text{Dose of drug into compartment S2} \times 100}{\text{Nominal dose}} \quad \text{Eq. 1}
\]

\[
\text{Effective Index} = \sqrt{(100-DF) \times \text{FPF}} \quad \text{Eq. 2}
\]

\[
\text{Effective index is the geometric mean of the total emitted dose (ED) and Fine Particle Fraction (FPF)}
\]

\[
\text{Where, DF=Device Fraction and FPF= Fine particle Fraction}
\]

\[
\text{Emitted fraction=} \frac{\text{Emitted dose of Drug (comp S1+ S2+ S3)}}{\text{Nominal Dose}} \times 100 \quad \text{Eq. 3}
\]

**Scanning Electron Microscopy (SEM)**

Scanning electron microscopy was used to study shape and surface texture of folate anchored andrographolide microparticles. Photomicrographs were taken by scanning electron microscope (Jeol, JSM 6360, Japan) at an original magnification of X1000 of plain drug and microparticles after spray drying.

**Fourier Transform Infrared Spectrophotometer Study (FTIR)**

The interaction between andrographolide and folic acid of physical mixture and spray dried sample was studied using FTIR. At the same time the association of chitosan with folic acid was achieved using FTIR spectrum (Jasco-V-530 model). About 2 mg of sample is ground thoroughly with KBr; uniformly mixed sample kept in sample holder and spectra was recorded over the wave number 400-4000 cm\(^{-1}\).11

**Powder X-ray diffraction study (PXRD)**

Crystalinity of plain drug and spray dried microparticles was checked by PXRD study. Sample is irradiated with monochromatic Cu Kα radiation (1.542 A\(^\)\) between 5° & 55°(using 2θ) on an X-ray diffractometer (Philips analytical XRD, PW 3710). The voltage & current applied were 40KV & 30 mA respectively.

**Differential Scanning Calorimeter (DSC)**

Thermal behavior of plain drug and spray dried microparticles was analysed by differential scanning calorimeter (TA instruments, model SDT 2960, USA) equipped with intracooler & refrigerated cooling system. Zinc metal or Calcium oxalate was used as standard
to calibrate the DSC temperature and enthalpy scale. Samples were hermetically sealed in an aluminum crucible. The system was purged with nitrogen gas at a flow rate of 60 mL/min. Heating was done from 10°C to 350°C at rate of 10°C/min.

**Biological activity (Angiogenesis)**

Angiogenesis is the physiological process involving the growth of new blood vessels which is essential for organ growth and repair. However, the imbalance in angiogenesis contributes to numerous pathologies like cancer. The chorioallantoic membrane (CAM) model allows one to evaluate the extent of angiogenesis, in the presence of growth stimulator such as calf serum.

Fertilized chicken eggs were incubated at 37°C at constant humidity and on 3rd incubation day, a square window was opened in the shell and 2 to 3 ml of albumin was removed to allow detachment of the developing chorioallantoic membrane (CAM). The window was sealed with a cover slip and the eggs were returned to the incubator. On 8th day, 1 μg of calf serum alone as positive control, and together with various doses of test compound, was implanted on top of the CAM. The sponge traps the sample and allows slow release of the product. CAM was examined daily until day 12, when the angiogenic response peaks. Photographed of ovo was captured by camera system13.

**MTT Assay**

MTT Assay was used to check the inhibitory effect of andrographolide on lung cancer, for that human lung cancer cell line (H522) was used. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Cells were seeded at a concentration 1.5x10⁴ cells/ml in a 96-well plate. After overnight incubation, serial concentrations of andrographolide (250, 500 and 1000 μg/ml) were added. Each concentration was repeated three times. These cells were incubated in a humidified atmosphere with 5% CO₂ for 24 hrs. Then, MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) solution (5.00 mg/ml) was added to each well and incubated at 37.8°C for 4 hrs14. The medium was removed and Formazan was dissolved in DMSO and the optical density was measured at 630 nm. The growth inhibition was determined as:

\[
\% \text{Growth inhibition} = \frac{\text{Sample optical density}}{\text{Control optical density}} \times 100 \quad \text{Eq. 4}
\]

**Stability Study**

The stability of spray dried microparticles, packed in aluminum foil was monitored up to 3 months at ambient temperature and relative humidity (30°C/65% RH). Samples were removed periodically (15 days, 1 month and 3 months) and characterized for drug content and dissolution studies in comparison with the initial sample.

**RESULT AND DISCUSSION**

**Percent Yield and Drug Content**

The % yield and drug content of different formulations of andrographolide microparticles showed in Table 1. From table 1, % yield of AC5 batch was found to be 78.34±3.95% and drug content of AC1 was found to be 10.36%.

<table>
<thead>
<tr>
<th>Batch Code</th>
<th>Andrographolide: Chitosan (AC)</th>
<th>% Yield</th>
<th>Drug Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>2:1</td>
<td>64.35±2.32%</td>
<td>10.36%</td>
</tr>
<tr>
<td>AC2</td>
<td>1:1</td>
<td>75.4±3.95%</td>
<td>8.58%</td>
</tr>
<tr>
<td>AC3</td>
<td>1:2</td>
<td>77.5±3.95%</td>
<td>5.74%</td>
</tr>
<tr>
<td>AC4</td>
<td>1:3</td>
<td>62.43±3.95%</td>
<td>4.05%</td>
</tr>
<tr>
<td>AC5</td>
<td>1:4</td>
<td>78.34±3.95%</td>
<td>2.94%</td>
</tr>
</tbody>
</table>

**In Vitro Dissolution Studies**

From the in vitro dissolution study (fig. 2) it observed that the release of andrographolide was extending up to 15 hrs which indicates extended release of drug from microparticle. Chitosan is an excipient which helps in extended release and dissolution enhancement of andrographolide. AC1 and AC2 batch containing less amount of chitosan thus they are contributing less to solubility enhancement of drug. While AC4 and AC5 contain high proportion of chitosan which enhances the dissolution at certain point and then decreases dissolution rate of drug. AC3 batch contain optimum concentration of Drug: Polymer ratio which help to increase the dissolution and shows extended release.

**In Vitro Deposition Study**

Fine particle fraction for AC1, AC2 and AC3 batch was about 26.65, 20.17 and 36.52 respectively and shown in table 2.

<table>
<thead>
<tr>
<th>Batch Code</th>
<th>% Fine Particle Fraction</th>
<th>Maximum Effective Index</th>
<th>% Assay Determined by UV Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>26.65</td>
<td>46%</td>
<td>4.17%</td>
</tr>
<tr>
<td>AC2</td>
<td>20.17</td>
<td>46%</td>
<td>4.17%</td>
</tr>
<tr>
<td>AC3</td>
<td>36.52</td>
<td>46%</td>
<td>4.17%</td>
</tr>
</tbody>
</table>

Fig. 2: Percentage drug release of spray dried microparticles

Fine particle fraction greater than 30% is limit for pulmonary application. Maximum Effective index was obtained 46%. The 5mg of therotical dose administered gives 4.17% of assay determined by UV method. From in vitro deposition data, batch AC3 was selected as...
optimized batch. It was selected for further preparation and evaluation of corrugated particles at different inlet temperature of spray dryer. (table 3)

Surface Morphology

From SEM analysis (fig. 3) surface morphology of microparticles was studied. Enhanced aerosol performance of powders can be obtained by surface modification of the particles. The surface asperities of the corrugated particles could lower the true area of contact between the particles, and thus reduce the powder cohesiveness. Optimized batch AC3 was further analyzed at different temperature for corrugated particles. Andrographolide exhibited flat needles of different sizes, broken and with well developed edges. SEM of the different temperature showed different sphericity of the particle and as the temperature decreases corrugated particle were obtained. Powder for pulmonary inhalation requires particle size within the range of 3 to 7 micron.

Table 2: Fine particle fraction for batch AC1 to AC5

<table>
<thead>
<tr>
<th>Batch Code</th>
<th>Drug: Polymer</th>
<th>Fine Particle Fraction</th>
<th>Effective Index</th>
<th>Emitted Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>2:1</td>
<td>26.65</td>
<td>40.0461</td>
<td>36.43%</td>
</tr>
<tr>
<td>AC2</td>
<td>1:1</td>
<td>20.17</td>
<td>38.9751</td>
<td>25.56%</td>
</tr>
<tr>
<td>AC3</td>
<td>1:2</td>
<td>36.52</td>
<td>46.3289</td>
<td>40.21%</td>
</tr>
<tr>
<td>AC4</td>
<td>1:3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AC5</td>
<td>1:4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 3: Optimized batch at different Spray drying parameter

<table>
<thead>
<tr>
<th>Batch Code</th>
<th>Inlet Temp</th>
<th>Outlet Temp</th>
<th>Aspiration Rate</th>
<th>%Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC3a</td>
<td>180</td>
<td>80</td>
<td>7 ml</td>
<td>75%</td>
</tr>
<tr>
<td>AC3b</td>
<td>160</td>
<td>80</td>
<td>7 ml</td>
<td>73%</td>
</tr>
<tr>
<td>AC3c</td>
<td>140</td>
<td>80</td>
<td>7 ml</td>
<td>71%</td>
</tr>
<tr>
<td>AC3d</td>
<td>120</td>
<td>80</td>
<td>7 ml</td>
<td>77%</td>
</tr>
</tbody>
</table>

Fig. 3: (A) SEM of Andrographolide at 1500X, (B) SEM of AC3a at temperature 180°C at 2000X, (C) SEM of AC3b at temperature 160°C at 2000X, (D) SEM of AC3c at temperature 140°C at 1500X, (E) SEM of AC3d at temperature 120°C at 1500X.
FTIR Study

FTIR spectroscopy was carried out for elucidation of the interaction between andrographolide and chitosan in the solid state in Fig. 4. The FTIR spectrum of pure andrographolide showed a characteristic C=O absorption band at 1674.36 cm\(^{-1}\) and an OH stretch at 3395.02 cm\(^{-1}\). Exocyclic methylene group was observed at 900 cm\(^{-1}\). The free -OH attached to the lactone ring demonstrated a band at 3386.39 cm\(^{-1}\). FTIR spectra of chitosan showed characteristics peaks of OH Stretch at 3480 and at 3559 cm\(^{-1}\). Characteristics peaks of NH stretch at 3459 cm\(^{-1}\). Peaks at 1662 indicating stretching because of C=O, 1160 cm\(^{-1}\) indicating characteristic s peaks of C-O-C. Folic acid showed observable peaks at 3640 cm\(^{-1}\) of OH stretching and 3467 cm\(^{-1}\) of NH stretching. The FTIR spectra of chitosan and folic acid demonstrated peaks at 3400-3500 cm\(^{-1}\) of NH stretching which get diminished in conjugation. Conjugation of folic acid and chitosan was done by using EDC demonstrated peaks of -NH associated band at 1588 cm\(^{-1}\), also a new bond at 1064 cm\(^{-1}\) was observed of C-N conjugation showed the disappearance of the NH bending in the primary amine and appearance of NH associated band at 1588 cm\(^{-1}\) of the NH bending in the secondary amine.

PXRD Study

The structure of andrographolide, isolated from Andrographis Paniculata Nees, has been established by means of a single crystal X-ray analysis. The characteristics peaks of andrographolide appeared at diffraction angle of 2θ at 21.52°, 24.65°, 29.03°, 33.27°, 38.74°, 60.26° etc. indicating that andrographolide present in crystalline form in Fig. 5. The absence of crystalline peaks attributable to andrographolide in all spray dried batches revealed that andrographolide crystals were transformed to an amorphous state which contributes to increased solubility of andrographolide.

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![Fig. 4: FTIR spectra of folic acid (red), chitosan (blue) and conjugation (green)](image1)

![Fig. 5: XRPD pattern of andrographolide, folic acid, chitosan, and microparticles of andrographolide (AC3)](image2)
DSC Thermograms

DSC thermogram of andrographolide showed an endotherm at 238.40°C and folic acid at 248.70°C in fig. 6. The spray dried microparticle shows slight shifting of melting endotherm at lower temperature indicates crystalline andrographolide was converted into amorphous form. The spray dried microparticle shows small endothermic transition due to transformation of its major component to amorphous state. The crystallization inhibition of andrographolide may also be attributed to hydrogen bonding between the drug and the polymer and the entrapment of drug molecules in the polymer matrix during spray drying.

![DSC Thermogram of Andrographolide, Folic Acid, Chitosan and AC3 Batch](image)

**Fig. 6:** DSC thermogram of andrographolide, folic acid, chitosan and AC3 batch Biological activity (Angiogenesis)

![Angiogenesis Study Images](image)

**Fig. 7:** A- Chicken egg chorioallantoic membrane on 4th day before injection, B- Chicken egg chorioallantoic membrane on 4th day before injection, C- Egg implemented with calf serum, A1- Chicken egg chorioallantoic membrane on 8th day after injection of pure drug, B1- chicken egg chorioallantoic membrane on 8th day after injection of AC3 Batch, C1- Egg implemented with calf serum.

In angiogenesis study, eggs were subjected to visual observation of chorioallantoic membrane (fig. 7) Enhancement in growth observed in the egg in which calf serum was injected. Complete inhibition of blood vessel growth was observed into the egg in which andrographolide was injected and also in which AC3 batch was injected.

**MTT assay**

Equivalent quantity of andrographolide and optimized batch AC3d were subjected to MTT assay to check the inhibitory effect on growth of lung cancer cells (H522).
Table 4: Percentage Growth inhibited of H522 by andrographolide and ACd3

As per table 4, cancer cell growth was inhibited by increased concentration of andrographolide but it was comparatively less than growth inhibited by andrographolide DPI. This indicates that spray dried andrographolide microparticles were having improved solubility than andrographolide drug only.

<table>
<thead>
<tr>
<th>Quantity(µg/ml)</th>
<th>% Growth Inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrographolide</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>25.23</td>
</tr>
<tr>
<td>250</td>
<td>29.41</td>
</tr>
<tr>
<td>500</td>
<td>34.29</td>
</tr>
<tr>
<td>1000</td>
<td>46.15</td>
</tr>
<tr>
<td>ACd3</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>31.74</td>
</tr>
<tr>
<td>250</td>
<td>42.42</td>
</tr>
<tr>
<td>500</td>
<td>49.82</td>
</tr>
<tr>
<td>1000</td>
<td>60.00</td>
</tr>
</tbody>
</table>

CONCLUSION

Folate anchored chitosan based andrographolide microparticles were successfully prepared by spray drying technique. Conjugation of folic acid with chitosan was confirmed by FTIR study. Subsequently, microparticles were formulated as a DPI by using lactose as a carrier. PXRD and DSC study exhibited decreased crystallinity and improved micromeritic properties. Optimized batch was confirmed by in vitro dissolution which showed 70% release with in 15 hr. contributed to decreasing dosing frequency. Twin impinger analysis studies showed (35%) % of fine particle fraction which can be attributed to corrugated nature of microparticles. Angiolytic and MTT assay studies revealed that, DPI form shows many fold increase in activity than plain drug.

ACKNOWLEDGEMENTS

We would like to thank Principal, Bharati Vidyapeeth College of Pharmacy, Kolhapur for providing facilities to carry out this work.

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