Dendrimers based on a polyester scaffold were shown to be nontoxic and previously hydroxyl- or methoxy-terminated proteins [7]. Dendrimers possess hydrophobic core and hydrophilic molecular weight distribution, scaffolding properties, large number of peripheral functionalities, and several unique properties such as nanoscale size, monodisperse molecular weight distribution, container like properties in solution [8]. In addition, dendrimers are hydrophilic on the exterior and they have shown to exhibit micelle like behavior and to target tumor cells more effectively than small molecules [9]. Dendrimers are a fourth new architectural class of polymers with dendritic architecture as a carrier for sustained release of poorly water soluble drugs. It is well known that low aqueous solubility of the new compound development [1]. Most part of the body is made up of both in vitro and in vivo [18]. This motivated us to evaluate our dendritic architecture as a carrier for sustained release of poorly water soluble drugs.

In the present work, we have developed full generation triazine based dendrimer G1(OH)8, G2(OH)32 and G3(OH)128 with 8, 32 and 128 hydroxyl groups on the periphery as a solubility enhancer and drug carrier for poorly water soluble drug ketoprofen. Effect of certain parameters such as pH, concentration and the generation on solubilisation of ketoprofen by dendrimer was studied. Ketoprofen was loaded in G3(OH)128 dendrimer by the inclusion complex technique and release of Ketoprofen from ketoprofen loaded dendrimer was measured and compared with free ketoprofen. Ketoprofen loaded dendrimer was characterized by Fourier Transform infrared spectroscopy. Sustained release of ketoprofen from ketoprofen loaded dendrimer was studied and compared to that of free ketoprofen. Cytotoxicity and hemolytic assay revealed that dendrimers were less toxic compared to PAMAM dendrimers.

Conclusion: Improved solubility of ketoprofen by dendrimer generations, its slow release from G3(OH)128 dendrimer and cytotoxicity and hemolytic assay showed dendrimers have potential as drug carriers.

Keywords: Triazine Based Dendrimer, Sustained Release, Cytotoxicity, Hemolysis, Ketoprofen, Encapsulation.
piperazine (0.01 mmol) at 0-5°C to give 1,4-bis(4,6-dichloro-1,3,5-triazin-2-yl)piperazine as core for dendrimer synthesis. 1,4-bis(4,6-dichloro-1,3,5-triazin-2-yl)piperazine was purified by washing with acetone and methanol. 1,4-bis(4,6-dichloro-1,3,5-triazin-2-yl)piperazine (0.01 mmol) was reacted with diethanolamine (0.16 mmol) to give chlorinated dendrimer G1(OH)32. The above two steps were repeated to give half generation G2.5 and third generation G3(OH)128 dendrimer. The theoretical surface groups of G1(OH)8, G2(OH)32 and G3(OH)128 dendrimers were fully characterized by spectral analysis such as, FT-IR, 1H-NMR, 13C-NMR and ESI-Mass spectrometry [12].

Solubility study
Solubility study was carried out according to the method described by Higuchi and Connors [19]. Excess of ketoprofen was added to screw-capped vials containing different concentrations (0.6 mmol to 3 mmol) of dendrimer generations in buffers of 4.0, 7.4 and 10 pH. Vials were shaken for 48 hours at 37°C in shaking water bath. The vials were centrifuged to remove undissolved ketoprofen and absorbance of ketoprofen was measured at its characteristic wavelength 260 nm using Shimadzu UV-1800 spectrophotometer.

Drug encapsulation
Generally there are two approaches for drug loading in dendrimer either by inclusion complex or by conjugation. In the present approach, we have utilized inclusion complex technique. Drug loading was performed by reported methods with little modifications [20, 22]. A known amount of ketoprofen was added to generation 3 dendrimer G3(OH)128 (3 mmol in 10 ml of distilled water) solution. The mixture was stirred for 72 hours at room temperature. The mixture was then filtered and 5 ml of methanol was passed through five times through the filter to remove excess of ketoprofen. Access Ketoprofen from filter and each fraction of methanol was analyzed by UV spectrophotometer to determine amount of encapsulated drug indirectly.

In vitro drug release [22]
Pure ketoprofen was dissolved in methanol (2 mg/ml) and used as control. The prepared ketoprofen loaded dendrimer was dissolved in distilled water at a concentration of 2 mg/ml (the same concentration of ketoprofen as 2 mg/ml pure drug solution). This solution (2 ml in volume) was transferred to a dialysis bag (size cut off = 2.5 nm) immediately. The dialysis bag was placed in a 50 ml beaker containing 40 ml distilled waters. The outer phase was stirred continuously. After a scheduled interval of time for 0.5 hours, 100 μl of sample was withdrawn from the outer phase, and the outer phase was again replenished with 100 μl distilled waters. The absorbance of the outer phase was monitored at 260 nm using a spectrophotometer in order to characterize the concentration of ketoprofen.

Hemolysis study [21]
About 5 ml of the human blood from healthy individual was collected in a tube containing heparin. The blood was centrifuged at 1500 RPM for 3 minutes. The supernatant (Erythrocyte) was collected and plasma was discarded. The pellet was washed 3 times using 0.75% NaCl and centrifuged at 1500 RPM for 5 mins. The cells were resuspended in normal saline to 0.5%. Washed erythrocytes were stored at 4°C and used within 6 hours for the hemolysis assay. To 0.5 ml of cell suspension, 0.5 ml of different concentration of test sample (40, 60, 80 and 100 μg/ml in phosphate buffer saline (pH 7.2)) was added and incubated for 1 hr. After centrifugation, the supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 540 nm. The phosphate buffer saline and distilled water were used as minimal and maximum hemolysis control.

Cytotoxicity study [21]
The monolayer cell culture was trypsinized and the cell count was adjusted to 3 Lac cells/ml using medium containing 10% fetal bovine serum. Pre incubate cells at a concentration of 1×106 cells/ml in culture medium for 3 hours at 37°C and 5% CO2. The cells were seeded at a concentration of 5×104 cells/well in 100 μl culture medium and incubated at 37°C in 5% CO2 incubator for 24 hrs. After 24 hours, when the monolayer formed, the supernatant was flicked off and added previously diluted with media of 100 μl of different concentrations of test extract in microtitre plates and kept for incubation at 37°C in 5% CO2 incubator for 48 hours and cells were periodically checked for granularity, shrinkage, swelling. After 48 hours, the sample solution in the wells was flicked off and 10μl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37°C in 5% CO2 incubator. The supernatant was removed 100 μl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at 570 nm.

Statistical analysis
Data are expressed as the mean, standard deviation (SD) of obtaining results. The statistical analysis of data was performed using analysis of variance (ANOVA) [Graphpad, Version 2.01, San Diego, CA]. A value of p<0.05 was considered as statistically significant.

RESULTS AND DISCUSSION
Synthesis of dendrimers

Table 1: Physical description of dendrimer generations

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Appearance</th>
<th>Solubility in water</th>
<th>Theoretical surface groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>C₈H₈Cl₁N₈</td>
<td>White solid</td>
<td>Insoluble</td>
<td>Cl(4)</td>
</tr>
<tr>
<td>G1(OH)₈</td>
<td>C₁₂H₁₄N₁₂O₈</td>
<td>Brown liquid</td>
<td>Soluble</td>
<td>OH(8)</td>
</tr>
<tr>
<td>G1.5</td>
<td>C₁₂H₁₄Cl₁₃N₉O₅</td>
<td>White solid</td>
<td>Insoluble</td>
<td>OH(16)</td>
</tr>
<tr>
<td>G2(OH)₃₂</td>
<td>C₁₄H₁₄Cl₁₃N₉O₅</td>
<td>Brown liquid</td>
<td>Soluble</td>
<td>OH(32)</td>
</tr>
<tr>
<td>G2.5</td>
<td>C₁₄H₁₄Cl₁₃N₉O₁₀</td>
<td>White solid</td>
<td>Insoluble</td>
<td>CH(64)</td>
</tr>
<tr>
<td>G3(OH)₁₂₈</td>
<td>C₁₆H₁₆Cl₁₃N₉O₁₈</td>
<td>Brown liquid</td>
<td>Soluble</td>
<td>OH(128)</td>
</tr>
</tbody>
</table>

Fig. 1: Structures of: i) G1(OH)₈, ii) G2(OH)₃₂ and c) G3(OH)₁₂₈ Dendrimer
Synthesis and characterization of s-triazine based dendritic generation G1(OH)₈, G2(OH)₃₂ and G3(OH)₁₂₈ [fig. 1] based on piperezine was already reported [12]. As shown in table 1, only full generation dendrimers G1(OH)₈, G2(OH)₃₂ and G3(OH)₁₂₈ were water soluble whereas half generation dendrimers and core compound were water insoluble. Therefore, only full generation dendrimers G1(OH)₈, G2(OH)₃₂ and G3(OH)₁₂₈ were utilized for drug solubilisation and drug delivery.

Drug encapsulation and characterization of ketoprofen loaded dendrimer

G3(OH)₁₂₈ dendrimers showed maximum solubilisation of ketoprofen, therefore sustained release study was carried out by using G3(OH)₁₂₈ dendrimer. Ketoprofen drug was loaded with G3(OH)₁₂₈ dendrimer by inclusion complex technique [20, 22]. It was observed from UV spectrometer that about 24.28% ketoprofen was loaded in G3(OH)₁₂₈ dendrimer. Ketoprofen loaded dendrimer was further characterized by FT-IR spectroscopy and compared with FT-IR spectra of pure G3(OH)₁₂₈ and ketoprofen drug. FT-IR spectrum of pure G3(OH)₁₂₈ dendrimer [fig. 3a] showed characteristic absorption bands 3356 cm⁻¹ for O-H stretching for hydroxyl groups, 1064 cm⁻¹ for C-O stretching of ether linkages, FT-IR spectrum of pure ketoprofen [fig. 3b] showed characteristic absorption bands at 3010 cm⁻¹, 2895 cm⁻¹ for aromatic C-H stretching, 1665, 1735 cm⁻¹ for carbonyl stretching. FT-IR spectrum of ketoprofen loaded dendrimer [fig. 3c] showed absorption band at 3377 cm⁻¹ for O-H stretching, at 2885, 2810 cm⁻¹ for C-H stretching, at 1785, 1615 cm⁻¹ for carbonyl stretching and at 1055 cm⁻¹ for C-O stretching. So, a little shift in bands for O-H stretching and carbonyl stretching was observed and other characteristic bands for both G3(OH)₁₂₈ dendrimer and ketoprofen remained unchanged. So, as a dendrimer contains a hydrophobic triazine ring in interior regions which may impart hydrophobic interaction and the hydroxyl groups in the exterior, which may impart hydrogen bonding so, dendrimer may have enhanced solubility of ketoprofen and their encapsulation by either hydrophilic interaction or hydrogen bonding or both [16, 17, 22].

Sustained release

It was noted that about 95% of ketoprofen were released within 2.5 hours from free ketoprofen. Whereas the same quantity of the drug was released after 6.5 hours from ketoprofen loaded dendrimer [fig. 4]. So, Ketoprofen loaded dendrimer releases ketoprofen slowly compared to free ketoprofen. However, release of ketoprofen was comparatively faster than PAMAM dendrimer which released about 76% ketoprofen in 10 hours [25].

Hemolytic potential

Hemolysis assay gives quantitative estimation about hemoglobin release when red blood cells are treated with dendrimers. The data obtained in such assay also give a qualitative indication of potential damage to RBCs of dendrimer administered. Hemolytic assay of dendrimer at different concentration 40, 60, 80, 100 µg/ml was carried out. The results are displayed in fig. 5. It was observed that G3(OH)₃₂ dendrimer showed concentration dependent hemolysis. However, triazine based G3(OH)₁₂₈ dendrimer were significantly less hemolytic compared to PAMAM dendrimer [23]. Positively charged amine groups of PAMAM dendrimer interacts with negatively charged surfaces of red blood cells and caused hemolysis [24]. In comparison, G3(OH)₃₂ dendrimers have an ionic hydroxyl groups on the surface which may have minimized interaction with red blood cells and displayed significantly less toxicity.

Cytotoxicity

Cytotoxicity of G3(OH)₁₂₈ dendrimer on A-549 cells was evaluated by MTT assay technique. The results are displayed in fig. 6. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)] is a yellow, tetrazolium salt. Metabolically active cells convert MTT into the dark blue water soluble formazan as a result of cleavage of tetrazolium ring. Formazan can be dissolved in a solvent and can be measured quantitatively. Our results [fig. 6] displayed that G3(OH)₁₂₈ dendrimer displayed more that 90% cell viability at concentration levels ranging from 10 µg/ml to 1000 µg/ml. G3(OH)₁₂₈ dendrimer was significantly less cytotoxic. Microscopic images [fig. 7. a-d] displays morphology of A-549 cell lines on when treated with control and different concentration of dendrimers is displayed, which showed a decrease in cell density with increase in dendrimer concentration from 10 µg/ml to 1000 µg/ml [26].
Fig. 3: a) IR spectrum of G3(OH)₁₂₈ dendrimer, b) FT-IR spectrum of ketoprofen and c) FT-IR spectrum of ketoprofen loaded G3(OH)₁₂₈ dendrimer

Fig. 4: % Cumulative release profile ketoprofen from free ketoprofen and ketoprofen containing G3(OH)₁₂₈ dendrimer (n=3)

Fig. 5: % Hemolysis of Red blood cells by G3(OH)₁₂₈ dendrimer after 1 hour of incubation (n=3)
REFERENCES
Declared None

CONFLICT OF INTERESTS
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