In vitro release and in vivo toxicity comparison studies between irinotecan and fullerene-biotin conjugated irinotecan

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ABSTRACT: The clinical use of irinotecan (IRI), one of the most commonly used antitumor drugs for colon cancer, is limited by its numerous side effects, and it also lacks selectivity and specificity for tumor cells. When conjugated with fullerenes and biotin, this drug may serve as a drug of choice to reduce these side effects. Fullerenes are an allotropic form of carbon with a closed cage-like structure that can act as a drug carrier, and the presence of biotin makes it a promising targeting agent. In this work, we performed release as well as toxicity comparison studies between pure irinotecan and fullerene-biotin-conjugated irinotecan. The release studies were performed by HPLC (High Performance Liquid Chromatography), while the toxicity studies were performed on a model of colon tumors in gopher rats. All animals were divided into four groups, namely normal, control, test and standard groups. Biochemical parameters and histopathological studies were performed to compare the acute toxicity of irinotecan and the conjugate (C60-PEI-biotin/IRI). It was found that IRI alone was unable to reduce the toxicity caused by DMH (1,2-dimethylhydrazine), whereas its conjugate was able to significantly reduce the toxic effects, which was also evident in the histopathological studies. Therefore, the study suggests that the administration of conjugated IRI reduces toxicity and proves to be a potential candidate for a preventive chemo effect.

KEYWORDS: Antitumor; irinotecan; toxicity; fullerene; conjugate.

1. INTRODUCTION

Irinotecan is an analog of camptothecin used as a first-line chemotherapeutic agent in the treatment of colorectal cancer [1]. It is a topoisomerase I inhibitor with a broad antitumor spectrum in the treatment of solid tumors. However, in addition to its efficacy, it also causes some serious toxic effects that require immediate insight into drug monitoring and reduction of these cytotoxic effects [2-5]. The toxicity of irinotecan is dose-dependent, but the observed effects are the same at all doses [6-9]. It also lacks tumor specificity and tumor selectivity, resulting in the killing of normal cells in addition to tumor cells. However, conjugation of irinotecan with fullerenes and biotin has been shown to be effective in reducing these toxicities [10]. Since fullerenes are an allotropic form of carbon, they have a closed cage-like structure that can make the conjugate a drug carrier [11-12]. On the other hand, biotin, a vitamin, provides target specificity to the conjugate because vitamin receptors are overexpressed on the surface of tumor cells [13-14]. This entire conjugate drug delivery system can act as a tumor-specific targeted drug delivery agent, which may lead to minimization of toxicity associated with irinotecan.

In this work, we performed a comparative study between the conjugated irinotecan (C60-PEI-biotin/IRI) and the pure irinotecan (Figure 1) to find out whether the conjugate is able to reduce the toxic effects induced by DMH (1,2-dimethylhydrazine) and proves to be more effective than the standard drug. The studies were conducted in Wistar albino rats in which 1,2-dimethylhydrazine was used to induce a colon tumor. Both the conjugate and the standard drug were administered intravenously to the DMH-induced tumor-bearing rats in separate groups. Toxicity was checked by examining liver, kidney and blood parameters of the rats. The same was confirmed by histopathological examination of liver and kidney at the end of tumor model. Using this in vivo antitumor model, it was confirmed that the conjugate is more
effective than the standard drug and thus can be used as an effective molecule in the treatment of colon cancer.

![Irinotecan Structure](image1)
![C60-PEI-Biotin/IRI Structure](image2)

Figure 1: Structures of irinotecan and fullerene-biotin conjugated irinotecan (IRI = Irinotecan, PEI = Polyethyleneimine)

2. RESULTS AND DISCUSSION

2.1 In vitro release study comparison

The release studies were performed in-vitro, in order to investigate the release kinetics of pure irinotecan and release of active drug from nanoparticle-drug system, for which we incubated the irinotecan and nanostructures in 50ml of PBS (phosphate buffered saline) solution and release assay was performed at 37°C with stirring rate of 100rpm (rotations per minute). Studies were conducted upto 30 hours for irinotecan, while for 144 hours in case of conjugate and during this time interval samples were withdrawn after a time gap from both systems.

![Release Profile](image3)

Figure 2: Release profile of a) IRI (Irinotecan) alone from PBS (phosphate buffered saline) solution b) Irinotecan from C60-PEI-Biotin/IRI nanosuspension
After 24 hours, the peak area for pure irinotecan was reached, but the release of the drug from the conjugate persisted up to 120 hours (Figure 2). By comparing these two results, we can conclude that the conjugate has a controlled release profile over a longer period of time, which was lower for irinotecan, which has also been mentioned in previous publications [15]. According to these results, the conjugate has been shown to be more effective than irinotecan alone and can release the drug over a longer period of time from a single dose. This may be further explored and prove to be a breakthrough choice. In addition, the kinetic order of drug release in both cases was found to be zero order by the graphical method.

2.2 Biochemical results

2.2.1 Liver function test

Liver toxicity can be confirmed by checking various liver enzymes or factors in the serum. These include the presence of enzymes such as transaminases [aspartate transaminase (AST) and alanine transaminase (ALT)] and phosphatase [alkaline phosphatase (ALP)] in serum, which reflect the state of liver tissue. The ALT enzyme, found in the cytoplasm and mitochondria, plays an important role in the gluconeogenesis pathway by converting alanine to pyruvate. On the other hand, AST acts as a transporter for reducing equivalents in mitochondria. ALP is another important hepatic marker enzyme, and an increase in serum ALP indicates pathological changes in bile flow. Routine monitoring of changes in ALP concentrations helps to determine liver toxicity. Due to DMH metabolism, the activity of transaminases in serum increases, leading to a loss of hepatocyte functionality, and the plasma membrane is also attacked by free radicals produced by liver cells. All of these lead to cell membrane disruption, and this liver damage results in intracellular enzymes entering the bloodstream and increasing their levels in case of toxicity [16-18]. In the current study, all these enzymes were analyzed in serum and it was found that the activity of AST, ALP and ALT enzymes were increased in serum of rats treated with DMH and DMH + IRI, while rats treated with C60-PEI-biotin/IRI showed a significant decrease in all these markers and thus had a better safety profile compared to irinotecan (Table 1).

<table>
<thead>
<tr>
<th>Group/Parameter</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>38.3±0.88</td>
<td>44.92±0.99</td>
<td>40.8±0.56</td>
</tr>
<tr>
<td>DMH</td>
<td>61.41±1.06</td>
<td>68.11±1.14</td>
<td>169.92±0.84</td>
</tr>
<tr>
<td>DMH+IRI</td>
<td>58.28±1.26</td>
<td>61.88±1.01</td>
<td>119.65±1.88</td>
</tr>
<tr>
<td>DMH+C60-PEI-Biotin/IRI</td>
<td>48.95±1.34</td>
<td>59.08±1.42</td>
<td>81.08±1.11</td>
</tr>
</tbody>
</table>

Table 1. Effect of DMH, DMH+IRI, DMH+C60-PEI-Biotin/IRI on liver function after 20 weeks of treatment

Units: IU/L; Data is expressed as Mean ±SD (n = 7). Data is analyzed using one-way ANOVA with keeping p≤0.05

(2.2.2) Kidney function test

Like in case of liver, several biochemical markers like creatinine, urea, and BUN (blood urea nitrogen) are used to detect any renal damage. Levels of these parameters get elevated on administration of DMH and irinotecan and it can tubular injury, which in turn affects reabsorption of water. If amino acid metabolism got affected, significant increase in the level of urea in serum was observed. In addition, the rise in urea and BUN may be attributed to an increase in nitrogen retention or extreme protein breakdown. In the current study, elevation in levels of creatinine, urea, and BUN in serum was observed in rats administered with DMH, leading to renal damage. DMH+IRI treated groups also showed mild elevated levels of these parameters. However, animals treated with C60-PEI-Biotin/IRI significantly lowered the levels of urea, creatinine, and BUN in rats, and this effect which can exist because fullerene and biotin both have antioxidant properties and this can help in free radical scavenging leading to protective effects on the renal injury caused by DMH treatment [19-21]. Therefore, results suggested less toxic effects of C60-PEI-Biotin/IRI conjugated in comparison to IRI (Table 2).
Table 2. Effect of DMH, DMH+IRI, DMH+C60-PEI-Biotin/IRI on kidney function after 20 weeks of treatment

<table>
<thead>
<tr>
<th>Group/Parameter</th>
<th>Urea</th>
<th>BUN</th>
<th>Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>26.45±1.2</td>
<td>13.6±0.85</td>
<td>3.41±0.55</td>
</tr>
<tr>
<td>DMH</td>
<td>43.52±1.71</td>
<td>25.65±1.24</td>
<td>5.71±0.82</td>
</tr>
<tr>
<td>DMH+IRI</td>
<td>38.74±1.11</td>
<td>19.22±0.83</td>
<td>5.10±0.64</td>
</tr>
<tr>
<td>DMH+C60-PEI-Biotin/IRI</td>
<td>29.62±1.96</td>
<td>14.07±1.01</td>
<td>5.08±0.60</td>
</tr>
</tbody>
</table>

*Units: Urea- mg/ml; BUN- mg/ml; Creatinine- mg/ml; Data is expressed as Mean ±SD (n = 7). Data is analyzed using one-way ANOVA with keeping p≤0.05 (BUN = Blood Urea Nitrogen, DMH = 1,2-Dimethylhydrazine, IRI = Irinotecan, PEI = Polyethyleneimine)*

2.2.3. Hematological parameters

A marked decrease in levels of blood cells was observed because of chemotherapy and this effect is particularly called as the “Nadir effect”. Rapidly dividing cells of the body get often affected by antitumor drugs and can cause drug-induced neutropenia which is a life-threatening side effect and also neutrophil count in blood get affected by these cytotoxic drugs [22-24]. By the above explanation, there was a significant decrease in the blood count of the animals administered with IRI; however, C60-PEI-Biotin/IRI treated animals showed improved blood count, which further suggested less toxicity of C60-PEI-Biotin/IRI in comparison to IRI (Table 3).

Table 3. Effect of DMH, DMH+IRI, DMH+C60-PEI-Biotin/IRI on hematological parameters after 20 weeks of treatment

<table>
<thead>
<tr>
<th>Group/Parameter</th>
<th>Hb</th>
<th>Platelets</th>
<th>TLC</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>13.1±0.5</td>
<td>64.27±1.82</td>
<td>828.14±1.77</td>
<td>71.71±2.28</td>
<td>35.42±2.37</td>
</tr>
<tr>
<td>DMH</td>
<td>10.06±1.41</td>
<td>64.5±1.35</td>
<td>906±2.3</td>
<td>75.35±1.30</td>
<td>27.7±0.88</td>
</tr>
<tr>
<td>DMH+IRI</td>
<td>8.29±0.44</td>
<td>47.77±0.93</td>
<td>540.57±3.6</td>
<td>69.07±1.06</td>
<td>29.37±0.74</td>
</tr>
<tr>
<td>DMH+C60-PEI-Biotin/IRI</td>
<td>12.17±0.64</td>
<td>49.14±0.72</td>
<td>554.42±2.22</td>
<td>76.81±1.48</td>
<td>26.61±0.47</td>
</tr>
</tbody>
</table>

*Units: Hemoglobin- g/dl, Platelets- x1000/µl, TLC- x10 counts, Neutrophils- %, Lymphocytes- %; Data is expressed as Mean ±SD (n = 7). Data is analyzed using one-way ANOVA with keeping p≤0.05. (Hb = Hemoglobin, TLC = Total leucocyte count, DMH = 1,2-Dimethylhydrazine, IRI = Irinotecan, PEI = Polyethyleneimine)*

2.3. Histopathological studies

A prominent central vein and normal lobular structure was seen in liver of normal rats confirming normal histo-architecture. The nuclei and cytoplasm showed usual staining. Structural disintegration of the hepatic plates was observed in DMH treated (control) rats along with dilated sinusoids and intracellular gaps were increased, also at certain regions neutrophil infiltration was visible. DMH+IRI group also showed damaged central vein and distorted hepatic lobules, hyperplasia of Kupffer cells, and atypical nuclei with vascular dilation in liver sections and it can be because of oxidative stress leading to liver inflammation. On the other hand, liver sections of DMH + C60-PEI-Biotin/IRI treated rats showed a decrease in number of degenerated hepatocytes, lower density of inflammatory cell infiltration, Kupffer cell activation and improved sinusoidal space in comparison to DMH+IRI group. Also, the biochemical parameters results were well correlated with the histopathological changes in the liver and all these findings suggested that the use of fullerene and biotin in combination with IRI can lower the side effects of the IRI, which can be due to their antioxidant properties and better take-up of conjugate by cancer cells in comparison to normal cells (Figure 3).
Figure 3: Histological observations of the liver induced with DMH and the effect of standard drug and conjugate treatment. (a) Normal morphology can be seen in liver section of group-I as central portal vein can be seen. (b) Sinusoidal dilation, necrosis and congestion seen in rats exposed to DMH (group 2) in liver section. (c) Conjugate (group 3) treated rats with mere cell infiltration have near to normal histoarchitecture (d) Hyperplasia and cell infiltration shown in liver of rats administered with irinotecan alone along with DMH.

In normal, healthy animals, the kidneys had a well-preserved glomerulus, and the tubular epithelial cells were also intact and had normal histo-architecture. However, mild tubulointerstitial damage was observed in DMH-treated rats. Both the standard and test groups exhibited extensive tubular epithelial cell loss, tubular dilatation, intratubular debris, and tubular cell atrophy. Inflammatory cell infiltration and an increase in interstitial area were observed in both the irinotecan and conjugate groups. However, tubulointerstitial damage was absent in the DMH+ C60-PEI-biotin/IRI-treated groups, indicating that the test conjugate was well tolerated by the carcinogenic rats (Figure 4).

Figure 4: Effect of DMH, Irinotecan, and C60-PEI-Biotin/IRI on histoarchitecture of kidney tissue after 20 weeks of treatment. (a) Haematoxylin and eosin stained kidney section of colon rats showing normal architecture (group 1). (b) DMH (1,2-dimethylhydrazine) alone exposed rats (group 2) kidney section showing tubulointerstitial injury (c) Conjugate (group 3) treated rats showed normal renal morphology with mere cell infiltration (d) Kidney of rats administered with irinotecan alone along with DMH induction showing tubular dilation, intratubular debris, and tubular cell atrophy.
3. CONCLUSION

When the release studies of irinotecan alone and fullerene-biotin-conjugated irinotecan were compared, it was found that the conjugate released the drug over a longer period of time (120 hours), whereas pure irinotecan was sustained for only 24 hours. This supports the use of the conjugate as a controlled drug delivery system. All biochemical parameters and histopathological studies in rats confirmed that the conjugate had lower toxicity in the liver and kidney compared with standard irinotecan. The tolerance of rats to the conjugate was greater than that of irinotecan, which may be due to biotin conjugation, as it is more likely to be taken up by cancer cells than by normal healthy cells. Oxidative stress has been shown to play a special role in cancer development, and compounds with antioxidant properties are widely used in tumor chemotherapy. Biotin is a well-known antioxidant vitamin, and its role in controlling oxidative stress has been explained in previous studies. In addition, we performed experiments with liver enzymes and found that the conjugate was able to decrease elevated levels of several liver enzymes, confirming the antioxidant effect of the conjugate [25-27]. This proves that the conjugate is more effective in treating cancer-related toxicities compared to pure irinotecan and that the controlled slow release profile of the conjugate makes it even more valuable.

4. MATERIALS AND METHODS

4.1. In vitro release study comparison between irinotecan and conjugate

For release study, both irinotecan and C₆₀-PEI-Biotin/IRI nanosuspension were placed into dialysis bag separately, which were dialyzed in 50 ml PBS solution. The release assays for both were performed at 37.0 ± 0.5°C with a stirring rate of 100rpm. The 0.2 milliliter of solution was withdrawn from PBS at various time intervals (0hrs, 6hrs, 12hrs, 24hrs, 30hrs for irinotecan and 12hrs, 24hrs, 48hrs, 72hrs, 96hrs, 120hrs, 144hrs in case of conjugate) and replaced by the same volume of fresh PBS solution after the withdrawal in both cases. The concentration of IRI released from pure irinotecan and C₆₀-PEI-Biotin into PBS solution was quantified using HPLC. It was done by using high performance liquid chromatography (HPLC, Cyberlab LC-100 series S-HPLC, USA) with following conditions: a C₈ column (5μm) having size 4.6mm×D. × 250mm; mobile phase [PBS: Methanol (MeOH): Acetonitrile (AcN) (55: 25: 20)]; column temperature 25°C; detection wavelength 254 nanometer; flow rate 1.0ml/min; and injection volume 20 microliter mobile phase [28].

The order for the release of drug form the nanosuspension was determined by applying graphical method of analysis, on the results obtained from the HPLC studies of the drug release. According to the method four graphs were plotted between concentration of the drug left in the nanosuspension vs time interval at which it was released and results were recorded on the following basis:

- If the plot of [C] vs t is a straight line, then the reaction follows zero-order.
- If the plot of log [C] vs t is a straight line, the reaction follows first-order.
- If the plot of t/[C] vs t is a straight line, the reaction follows second order.
- If the plot of 1/[C]^2 is a straight line, the reaction follows third order.

Here [C] is the concentration of suspension at any given time of the reaction (other t =0). [C] = (c-x) where c is the initial concentration and x is the amount of drug release from nanosuspension at time t. Among all these graphs, straight line will be obtained only in one case and that graph confirmed the order of reaction [29].

4.2. In vivo toxicity studies

All animal experiments were performed according to the protocol approved by IAEC (MMCP/IAEC/16) with ethical approval number IAEC/19/13. The Wistar Albino rats were obtained from the National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, Mohali. The colon tumors were induced in Wistar Albino Rats (males) by subcutaneous injection of 1,2 dimethylhydrazine (DMH; 15mg/kg/week for 5 weeks) in the right shoulder (140-200g). All animals were divided into four groups (7 animals per group), with minimum weight differences; group I normal group, group II control group (DMH), group III (DMH + C₆₀-PEI-Biotin/IRI (25mg/kg), and group IV (DMH + IRI; IRI dose: 25mg/kg), group IV untreated animals. The administrated dose given to the animals for conjugate testing was decided on the basis of toxicity studies performed in accordance with literature [30, 31]. Irinotecan was used as a standard to compare the results of C₆₀-PEI-Biotin/IRI suspension. The subcutaneous injection was used as a medium for giving the treatment, and the doses were given once a week for both test and standard
group until 20 weeks from the start of the study. Blood samples were taken and animals were sacrificed to carry out their serological studies by using standard kits and histological studies (liver, and kidney), respectively. The histopathological studies of isolated kidney, and liver tissues were also carried out to compare the toxicity of C60-PEI-Biotin/IR and IR [32].

4.2.1 Biochemical estimations

Liver function test:

Alkaline Phosphatase (ALP)

Alkaline Phosphatase enzyme is found in the liver, a form of hydrolase enzyme, helps in removing phosphate groups from various biomolecules (proteins, nucleotides, and alkaloids). ALP activity was estimated in serum by using ENZOPAK ALP kit (CC1-ALK.02M, 50x1.1, 16AX02M) obtained from Reckon Diagnostic P. Ltd. (India). The kit is based on the hydrolysis of p-nitrophenyl phosphate into chromogenic compound p-nitrophenol. The rate of increase in absorbance of the reaction mixture at 405nm due to liberation of p-nitrophenol is directly proportional to the alkaline phosphatase activity (Units obtained for ALP activity = IU/L) [33].

Alanine Transaminase (ALT)

Metabolism of amino acids occurs in liver by Alanine transaminase enzyme and increase in there levels from normal indicate presence of liver damage. ALT level was estimated in serum using ENZOPAK ALT kit (CC2-ALT.17N, 5x25 ml, B101311) obtained from Reckon Diagnostic Pvt. Ltd. (India). The kit was designed according to the described procedure of Henry et al., (1960). The assay included two step reaction in which pyruvate produced upon ALT or SGPT (Serum glutamate pyruvate transaminase) incubation was reduced by lactate dehydrogenase (LDH). The reduction was facilitated with the oxidation of NADH to NAD. The decrease in the absorbance of NADH at 340 nm was proportional to ALT activity (Units obtained for ALT activity = IU/L) [34].

Aspartate Transaminase (AST)

Aspartate transaminase is another liver enzyme which helps in metabolism of amino acids and as well can act as transporting enzyme in converting amino acids. AST level was estimated in tissue homogenate and serum using ENZOPAK AST kit (CC2-AST.16N, 5x25 ml, B071927) obtained from Reckon Diagnostic P. Ltd. (India). The kit was designed according to the described procedure of Karmen et al., (1955). The assay included two step reaction in which oxaloacetate produced upon serum glutamate oxaloacetate transaminase (SGOT/AST) incubation was reduced by malate dehydrogenase (MDH). The reduction was facilitated with the oxidation of NADH to NAD. The decrease in the absorbance of NADH at 340 nm was proportional to AST activity (Units obtained for AST activity = IU/L) [35].

Kidney function markers: Kidney function tests viz. Urea, creatinine, and blood urea nitrogen were performed and calculated with commercially available kits (Creatinine; CC3-CRE.08M, 120 ml, B111229 and for Urea and BUN; CC2-UAB.019, 5x10 ml, B041128) from Reckon diagnostics.

Creatinine

Creatinine is obtained as an end product in metabolism reaction and its amount depend on intake of dietary protein. In case of intense muscular activity there levels get affected and serum concentration of creatinine gets elevated in bloodstream (Units obtained for Creatinine = mg/ml) [36].

Urea

Metabolism of urea and nitrogen occurs in kidneys and it is also known as renal nitrogen metabolism and is required for healthy being. Any waste nitrogen gets converted into ammonia, and on further metabolism form urea which gets excreted. But in the presence of any kidney disease or renal failure there levels get elevated from normal, as kidney functions get impaired (Units obtained for Urea and BUN = mg/ml) [37].

Hematological parameters: Blood sample (100μl) was collected from the retro-orbital plexus of mice using fine glass capillaries in sterile eppendorf tubes. Tubes containing blood were allowed to clot at 37°C for 4 hours. The clotted blood was centrifuged at 3000 rpm for 10 minutes and the upper clear layer (serum) was
aspirated in another sterile eppendorf tube. Serum samples were stored at -80°C (Units: Hemoglobin- g/dl, Platelets- x1000/μl, TLC- x10 counts, Neutrophils- %age, Lymphocytes- %age) [38].

4.2.2 Histopathological estimations

To evaluate the histological alterations, tissues from normal, control as well as from treatment groups were subjected to Haematoxylin and Eosin (H&E) staining. Tissue sections (liver and kidney) were washed with normal saline and were immediately fixed in 10% formalin for about 24 hours. After fixation, tissues were dehydrated in ascending grades of alcohol (30%, 50%, 70%, 90%, and 100%) for an hour each. Samples were then kept in alcohol + benzene mixture (1:1) for 30-45 min, followed by benzene for 30 min and then embedded in a mixture of benzene and paraffin wax (1:1) for 1 hour at 58-60°C. Before proceeding for final embedding in the wax, the samples were immersed in pure molten wax with two changes of 3 hours each and were obtained as solid blocks, thereafter, 5-7 microns thick paraffin sections were cut with the help of hand-driven microtome and then placed on clean glass slides.

The sections so obtained were then dewaxed in xylene, rehydrated in descending series of ethanol (100%, 90%, 70%, 50%, and 30%), brought to water and stained in hematoxylin for 30 seconds. Slides were further treated with ascending series of ethanol (30%, 50%, and 70%) and were stained with alcoholic eosin for 1-2 min. The stained tissue sections were further differentiated with 90% ethanol and washed with absolute alcohol for 1 min each. The sections were finally cleared by rinsing the slides in xylene followed by mounting in DPX (Humanson, 1961). The tissue sections were analyzed under a light microscope (LEICA DM 3000) [39].

4.4. Statistical analysis

The results are expressed as mean ± SD (standard deviation) of seven animals in each group. For statistical significance, the data were analyzed using one-way ANOVA (Analysis of Variance). P values <0.05 were considered statistically significant.

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Conflict of interest statement: Authors do not have any conflict of interest with anyone.

REFERENCES


[34] Banda JM, Musa BOP, Onyemelukwe GC, Shittu SO, Babadoko AA, Bakari AG, Mamman AI, Sarkin-Pawa A, Junaid SA. T lymphocyte subpopulations in normal pregnancies and those complicated by eclampsia in Kaduna State, Nigeria. Open J Immunol. 2016; 6(3). [CrossRef]


[37] Schrier RW. Renal and electrolyte disorders: Lippincott Williams & Wilkins; 2010.


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