Research Article

Establishment of *in vitro – in vivo* correlation of antihyperlipidemic activity

U. S. Jijith[1,2], S. Jayakumari[1]*

ABSTRACT

Objective: In the proposed studies, we aimed to establish the *in vitro–in vivo* correlation (IVIVC) of antihyperlipidemic activity. We developed new *in vitro* chicken liver assay method for the evaluation of antihyperlipidemic activity and by using this method also evaluated atorvastatin antihyperlipidemic activity. In the proposed study, we aimed to correlate the *in vivo* response (serum total cholesterol) with an *in vitro* response (concentration of an extract of liver homogenate in isopropyl alcohol). **Materials and Methods:** Then, we hypothesized that chicken liver could be tried as an alternative to rat liver to avoid ethical issues. Moreover, chicken liver is widely available. Thus, we decided to include chicken liver homogenate as the source of enzymes for the *in vitro* biosynthesis of cholesterol homogenate with and without atorvastatin. We were selected n-hexane:isopropyl alcohol organic mixture to extract the lipid content from chicken liver. Isopropyl alcohol was used to make up the final volume before testing cholesterol content in residue. **Results:** Both *in vitro* and *in vivo* method results showed antihyperlipidemic activity and found that new apparatus is worth for screening activities. **Conclusions:** A good correlation was obtained between *in vitro* and *in vivo* data. Thus, it was concluded that an IVIVC was established for antihyperlipidemic activity.

KEY WORDS: Atorvastatin, Chicken liver, Cholesterol, HMG-CoA, Isopropyl alcohol, Lanosterol, N-hexane

INTRODUCTION

In vivo studies in rats are commonly employed for the screening of antihyperlipidemic drugs.[1] The use of this method suffers the disadvantages of animal usage and ethical issues. In this context, we hypothesized an *in vitro* system which would avoid such disadvantages.[1-6]

We proposed a new method named chicken liver assay for the *in vitro* evaluation of antihyperlipidemic agents. In the proposed study, we aimed to correlate the *in vivo* response (serum total cholesterol) with an *in vitro* response (concentration of an extract of liver homogenate in isopropyl alcohol). In the *in vitro* method, the cholesterol content of the liver homogenate after extraction (with 50 mL of a mixture of n-hexane:isopropyl alcohol in the ratio 3:2) and final volume makeup to 10 mL (using isopropyl alcohol) was determined.[7-12]

In order to have a same dose value for the purpose of correlation, we selected the *in vitro* atorvastatin dose as 0.18, 0.36, and 0.72 mg per g weight of liver homogenate. This well corresponds to the *in vivo* dose of 0.18, 0.36, and 0.72 mg per kg weight of rat.

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MATERIALS AND METHODS

Materials

Sodium acetate was from Astron chemicals, Ahmedabad, India. Isopropyl alcohol was supplied by Universal Chemical and Scientific Industries, Haripad, India. n-Hexane was purchased from Spectrum Reagents and Chemicals Pvt. Ltd., Cochin, India. Atorvastatin calcium was obtained as a gift sample from Dr. Reddy's Laboratories, Hyderabad, India. Lanosterol was purchased from Sisco Research Laboratories Pvt. Ltd, India. All other chemicals and reagents used were of analytical grade.

Methods

Preliminary studies of the proposed method – chicken liver assay

During our constant and extensive search for a suitable *in vitro* model, we came across the rat liver models for the study of cholesterol biosynthesis[13] in the study.

[1]Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels Institute of Sciences, Technology and Advanced Studies, Chennai, Tamil Nadu, India, [2]Department of Pharmacology, College of Pharmaceutical Sciences, Government Medical College, Kozhikode, Kerala, India

***Corresponding author:** Dr. S. Jayakumari, School of Pharmaceutical Sciences, Vels Institute of Sciences, Technology and Advanced Studies, Pallavaram, Chennai - 600 117, Tamil Nadu, India. Phone: 09840282617. E-mail: nisajayaa@gmail.com

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Our next objective was to evaluate the suitability of chicken liver in the evaluation of an antihyperlipidemic agent. For this purpose, we incubated the chicken liver. Then, we hypothesized that chicken liver could be tried as an alternative to rat liver to avoid ethical issues. Moreover, chicken liver is widely available. Thus, we decided to include chicken liver homogenate as the source of enzymes for the *in vitro* biosynthesis of cholesterol homogenate with and without atorvastatin, and finally, the cholesterol content of the liver homogenate after extraction (with 50 mL of a mixture of n-hexane:isopropyl alcohol in the ratio 3:2) and final volume makeup to 10 mL (using isopropyl alcohol) was determined.

Study of the effect of sodium acetate in the proposed method

Freshly isolated chicken liver was collected, chilled, and homogenized. The obtained liver homogenate was transferred into two iodine flasks with 17 g of the homogenate in each flask. Then, 1 mL an aqueous solution of sodium acetate (100 mg/mL) was added to sample A2 and mixed well [Table 1]. In addition to this, Krebs buffer was added to the homogenized samples for facilitating proper mixing. The homogenate was then incubated in a shaker water bath at a temperature of 37ºC for 4 h with or without atorvastatin added to it. After completion of the incubation period, the homogenate maintained in the iodine flask was treated with a mixture of n-hexane:isopropyl alcohol (50 mL) prepared in the ratio 3:2. The extraction using this solvent mixture was carried out overnight in a shaker water bath. During incubation, the stopper of the iodine flask was kept secured to avoid escape of the solvent by evaporation. After completion of the extraction using the solvent mixture, the sample was filtered using a filter paper. The filtrate was collected, and the aqueous phase (less in quantity) was separated and discarded. The organic phase containing the extracted lipids was then evaporated to dryness at room temperature. The dried residue was dissolved in isopropyl alcohol under magnetic stirring at 600 rpm and finally made up to 10 mL using isopropyl alcohol. The obtained sample was estimated for cholesterol content using Transasia ERBA® kit.

Study of the effect of atorvastatin

The method involves incubation of 10 µL of the sample with 1 mL of the reagent for 10 min. The enzymatic degradation of cholesterol to a purple-colored product is measured in comparison to a standard provided in the kit. Freshly isolated chicken liver was collected, chilled, and homogenized. The obtained liver homogenate was transferred into two iodine flasks with 17 g of the homogenate in each flask. Then, 1 mL an aqueous solution of sodium acetate (100 mg/mL) was added to samples B2 and B4 and mixed well [Table 2]. In addition to this, Krebs buffer was added to the homogenized samples for facilitating proper mixing. The homogenate was then incubated in a shaker water bath at a temperature of 37ºC for 4 h with or without atorvastatin added to it. After completion of the incubation period, the homogenate maintained in the iodine flask was treated with a mixture of n-hexane:isopropyl alcohol (50 mL) prepared in the ratio 3:2. The extraction using this solvent mixture was carried out overnight in a shaker water bath. During incubation, the stopper of the iodine flask was kept secured to avoid escape of the solvent by evaporation. After completion of the extraction using the solvent mixture, the sample was filtered using a filter paper. The filtrate was collected, and the aqueous phase (less in quantity) was separated and discarded. The organic phase containing the extracted lipids was then evaporated to dryness at room temperature. The dried residue was dissolved in isopropyl alcohol under magnetic stirring at 600 rpm and finally made up to 10 mL using isopropyl alcohol. The obtained sample was estimated for cholesterol content using Transasia ERBA® kit. The method involves incubation of 10 µL of the sample with 1 mL of the reagent for 10 min. The enzymatic degradation of cholesterol to a purple-colored product is measured in comparison to a standard provided in the kit.

In vitro studies using the developed chicken liver assay method

Freshly isolated chicken liver was collected, chilled, and homogenized. The obtained liver homogenate was transferred into four iodine flasks with 20 g of the homogenate in each flask. Then, 1 mL an aqueous solution of sodium acetate (100 mg/mL) was added

Table 1: Details of sample used to evaluate the effect of sodium acetate

Sample code	Liver homogenate (g)	Krebs buffer (mL)	Sodium acetate (mg)
A1	17	6	Nil
A2	17	5	100

Table 2: Sample used to study the effect of atorvastatin on acetate-mediated cholesterol biosynthesis in the chicken liver homogenate

Sample code	Liver homogenate (g)	Krebs buffer (mL)	Sodium acetate (mg)	Atorvastatin (mg)
B1	17	6	Nil	Nil
B2	17	5	100	Nil
B3	17	6	Nil	20
B4	17	5	100	20

Table 3: Details of sample used in the *in vitro* evaluation by chicken liver assay

Sample code	Liver homogenate (g)	Sodium acetate (mg)	Atorvastatin (mg) Dose×20 g
C1	20	100	Nil
C2	20	100	3.6
C3	20	100	7.2
C4	20	100	14.4

Table 4: Treatment plan for antihyperlipidemic study

Group	Name of group	Number of rats	Treatment
1	Normal control	6	No treatment
2	Hyperlipidemic control	6	Control
3	Hyperlipidemia induced	6	Standard antihyperlipidemic drug-atorvastatin (0.18 mg/kg)
4	Hyperlipidemia induced	6	Standard antihyperlipidemic drugs atorvastatin (0.36 mg/kg)
5	Hyperlipidemia induced	6	Standard antihyperlipidemic drugs-atorvastatin (0.72 mg/kg)
Total		30	-

to it and mixed well. In addition to this, Krebs buffer (5 mL) was added to the homogenate for facilitating proper mixing. The homogenate was then incubated in a shaker water bath at a temperature of 37ºC for 4 h with or without atorvastatin added to it. The samples are described in detail in Table 3. After completion of the incubation period, the homogenate maintained in the iodine flask was treated with a mixture of n-hexane:isopropyl alcohol (50 mL) prepared in the ratio 3:2. The extraction using this solvent mixture was carried out overnight in a shaker water bath. During incubation, the stopper of the iodine flask was kept secured to avoid escape of the solvent by evaporation. After completion of the extraction using the solvent mixture, the sample was filtered using a filter paper. The filtrate was collected, and the aqueous phase (less in quantity) was separated and discarded. The organic phase containing the extracted lipids was then evaporated to dryness at room temperature. The dried residue was dissolved in isopropyl alcohol under magnetic stirring at 600 rpm and finally made up to 10 mL using isopropyl alcohol. The obtained sample was estimated for cholesterol content using Transasia ERBA® kit. The method involves incubation of 10 µL of the sample with 1 mL of the reagent for 10 min. The enzymatic degradation of cholesterol to a purple-colored product is measured in comparison to a standard provided in the kit.

In vivo studies

The rats were randomly divided into five groups with 6 rats in each group. For inducing hyperlipidemia, a diet consisting of 200 mg cholesterol in 2 mL coconut oil was administered by oral route at a fixed convenient time for 14 days for Groups 2, 3, 4, and 5. Two hours after the diet, the standard drug atorvastatin was given at different dose for Groups 3, 4, and 5. On the 15th day, 2 mL blood was collected from each animal by retro-orbital puncturing under topical anesthesia (proparacaine eye drops 0.5%–1% drop per eye). The collected blood sample was mixed with anticoagulant properly and centrifuged at 5000 rpm for 20 min. The plasma was separated and stored at −21ºC until estimation of total cholesterol. Serum was assayed using standard diagnostic kit and determined the total cholesterol.

Dose Calculation for the Rats

Dose for the rats will be calculated after taking into consideration surface area ratio of a rat to that of a human being. The dose to be given to a 200 g rat on the basis of surface area ratio is determined by multiplying the human dose by a factor of 0.018.

- 10 mg is the dose of atorvastatin for adult male
- Therefore, for 200 g rat, atorvastatin dose will be 0.18 mg
- Atorvastatin dose, equivalent to atorvastatin 10 mg for adult human being, for rats will be 0.90 mg/kg body weight.

Based on the above dose calculation, in the present study, we selected three different doses of 0.18, 0.36, and 0.72 mg/kg. The detailed treatment plan is given in Table 4.

In vitro–in vivo correlation

Based on our *in vitro* and *in vivo* data, we further proceeded to correlation of these data. In order to have a same dose value for the purpose of correlation, we selected the *in vitro* atorvastatin dose as 0.18, 0.36, and 0.72 mg per g weight of liver homogenate. This well corresponds to the *in vivo* dose of 0.18, 0.36, and 0.72 mg per kg weight of rat.

RESULTS AND DISCUSSION

Preliminary Studies of the Proposed Method – Chicken Liver Assay

The above result made us think of a suitable precursor that would contribute to an increase in cholesterol content of the liver homogenate on incubation. It would be thus easy to study the

in vitro effect of an antihyperlipidemic agent such as atorvastatin. At this time, we came across one paper which describes the study of biosynthesis of cholesterol from lanosterol as a precursor.[13] Thus, we carried out evaluation of the utility of lanosterol as a precursor. We incubated the chicken liver homogenate with and without lanosterol and atorvastatin, and finally, the cholesterol content of the liver homogenate was determined. This time also, the observed results were not promising. We could not identify any significant inhibition of cholesterol level by atorvastatin. On a further extensive literature search, we identified that atorvastatin acts in a step previous to the formation of lanosterol. Thus, we concluded that lanosterol is not a suitable precursor for our study.

Meanwhile, we noticed the use of acetic acid and sodium acetate as precursor for the biosynthesis of cholesterol in isolated rat liver.[14,15] Furthermore, the HMG-CoA reductase enzyme is involved in the conversion of acetate to mevalonate, a step in the biosynthesis of cholesterol. Thus, we finally decided to choose sodium acetate as a precursor for the biosynthesis of cholesterol using chicken liver homogenate.

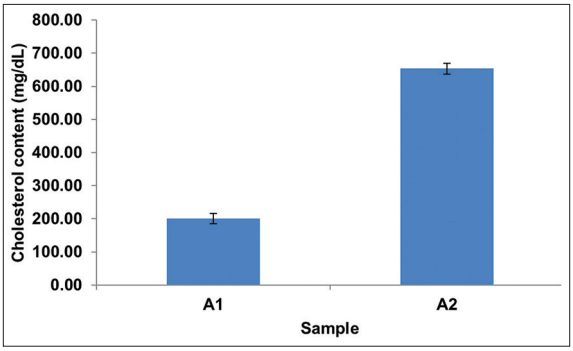

Figure 1: Cholesterol content after incubation of liver homogenate

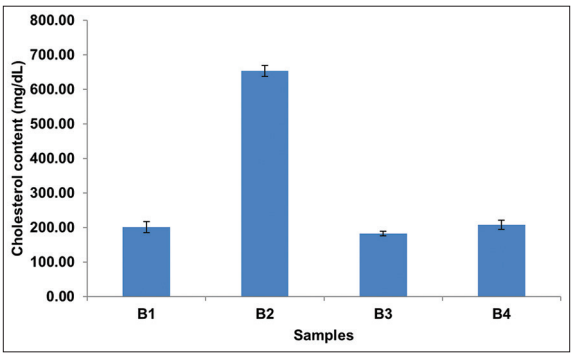

Figure 2: Effect of atorvastatin on acetate-mediated cholesterol biosynthesis in the chicken liver homogenate

Table 5: Experimental data of the study of effect of sodium acetate

Sample code	Cholesterol content in the final solution (mg/dL)
A1	200.67±15.95
A2	653.33±16.20

A rapid and simple method for the extraction of cholesterol from liver tissue was developed by modification of a reported method.[16]

Study of the Effect of Sodium Acetate in the Proposed Method

From the results of the study of the effect of sodium acetate, we decided to confirm the utility of sodium acetate as a precursor of cholesterol in the proposed chicken liver assay. For the purpose of evaluation, experiments were carried out with and without sodium acetate.

The obtained results were promising and demonstrated the utility of sodium acetate as a precursor for the formation of cholesterol [Table 5 and Figure 1]. It was noted that the mean cholesterol content after incubation of liver homogenate with sodium acetate was 653.33 ± 16.20 mg/dL. This was significantly higher than the cholesterol content after incubation of liver homogenate without sodium acetate (200.67 ± 15.95 mg/dL).

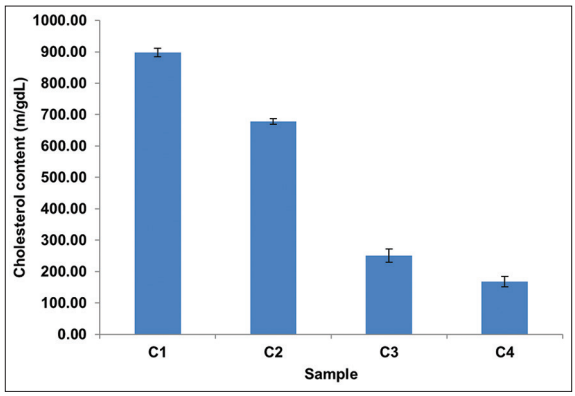

Figure 3: Cholesterol content during *in vitro* evaluation by chicken liver assay

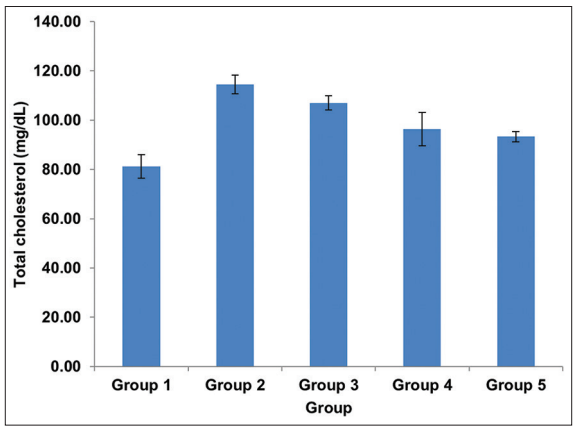

Figure 4: Response obtained for *in vivo* antihyperlipidemic studies

Study of the Effect of Atorvastatin

Interestingly, as expected, atorvastatin demonstrated inhibitory effect on acetate-mediated cholesterol biosynthesis in the chicken liver homogenate [Table 6 and Figure 2]. It is clear from the obtained result that atorvastatin, when present in the liver homogenate during incubation, inhibited the cholesterol biosynthesis and limits the cholesterol content to 207.33 ± 13.20 mg/dL, a value very near to the cholesterol content without sodium acetate (B1, 200.67 ± 15.95 mg/dL). The inhibition was significant when compared with the liver homogenate sample incubated with sodium acetate (B2, 653.33 ± 16.20 mg/dL). It is further noted that atorvastatin reduces the cholesterol content when incubated with atorvastatin. This means that some level of cholesterol biosynthesis occurs even without the presence of acetate. Nevertheless, in our preliminary studies, we have observed that this biosynthesis is not significant and an accurate effect is not observed. Thus, addition of sodium acetate as a precursor is absolutely necessary for the evaluation.

Table 6: Data for the effect of atorvastatin on acetate-mediated cholesterol biosynthesis in the chicken liver homogenate

Sample code	Cholesterol content in the final solution (mg/dL)
B1	200.67±15.95
B2	653.33±16.20
B3	182.67±06.81
B4	207.33±13.20

Table 7: Data for the *in vitro* evaluation by chicken liver assay

Sample code	Cholesterol content in the final solution (mg/dL)
C1	898.33±13.50
C2	678.33±08.74
C3	250.67±20.65
C4	168.00±16.09

In Vitro Studies using the Developed Chicken Liver Assay Method

From the results obtained for the *in vitro* evaluation by chicken liver assay [Table 7 and Figure 3], it was clear that the method was successful in the evaluation of atorvastatin for its antihyperlipidemic activity. A dose-dependent effect on the cholesterol content was observed for atorvastatin. Thus, it was concluded that the developed *in vitro* method was successful in the evaluation of antihyperlipidemic activity of atorvastatin.

In Vivo Studies

The results of *in vivo* studies are presented in Table 8 and Figure 4.

- $P < 0.001$ (Group 1 vs. Group 2; Group 1 vs. Group 3; Group 1 vs. Group 4; Group 1 vs. Group 5; Group 2 vs. Group 4; Group 2 vs. Group 5; Group 3 vs. Group 5)

Table 8: Data obtained for *in vivo* antihyperlipidemic studies

Group	Name of group	Treatment	Serum cholesterol content (mg/dL), mean±SD
1	Normal control	No treatment	81.17±4.79
2	Hyperlipidemic control	Control	114.50±3.78
3	Hyperlipidemia induced	Standard antihyperlipidemic drug - atorvastatin (0.18 mg/kg)	107.00±2.90
4	Hyperlipidemia induced	Standard antihyperlipidemic drugs - atorvastatin (0.36 mg/kg)	96.33±6.80
5	Hyperlipidemia induced	Standard antihyperlipidemic drugs - atorvastatin (0.72 mg/kg)	93.33±2.07

SD: Standard deviation

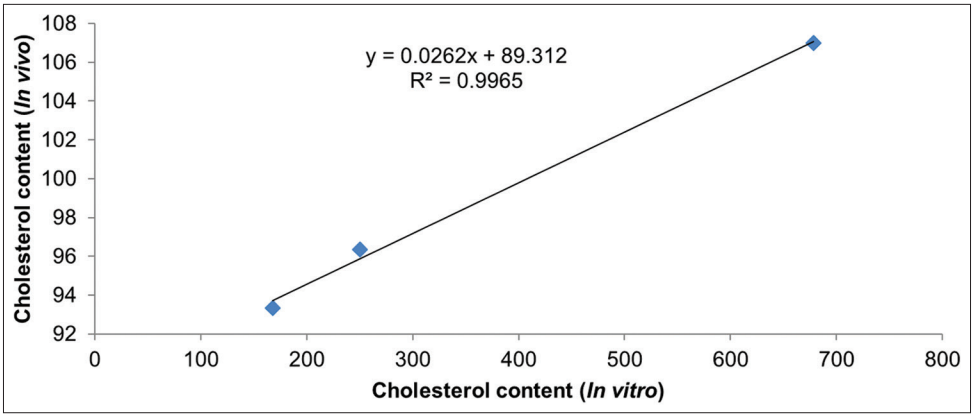

Figure 5: *In vitro – in vivo* correlation plot for cholesterol content

Table 9: Data for in vitro and *in vivo* correlation

Atorvastatin dose		Cholesterol content (mg/dL), mean±SD	
***In vitro* (mg/g)**	***In vivo* (mg/kg)**	***In vitro***	***In vivo***
0.18	0.18	678.33±08.74	107.00±2.90
0.36	0.36	250.67±20.65	96.33±6.80
0.72	0.72	168.00±16.09	93.33±2.07

SD: Standard deviation

- $P < 0.01$ (Group 3 vs. Group 4)
- $P < 0.05$ (Group 2 vs. Group 3)
- $P > 0.05$ (Group 4 vs. Group 5).

In Vitro – *In Vivo* Correlation

Table 9 shows the data used for correlation. Figure 5 displays the IVIVC plot. Interestingly, the IVIVC plot showed excellent correlation between *in vitro* and *in vivo* data with a $R^2 = 0.996$. Thus, it was concluded that the *in vitro* and *in vivo* data of cholesterol content could be used for correlation and prediction of *in vivo* response. Thus, a correlation between *in vitro* and *in vivo* data was established. From the obtained plot, we can easily predict the total serum cholesterol from the *in vitro* data obtained using the developed chicken liver assay method.

CONCLUSIONS

The developed *in vitro* method was useful in evaluating the antilipidemic activity of atorvastatin. The *in vivo* response of the selected dose of atorvastatin was evaluated in rats. A good correlation was obtained between *in vitro* and *in vivo* data. Thus, it was concluded that an IVIVC was established for antihyperlipidemic activity.

REFERENCES

1. Jijith US Jayakumari S. Screening methods for antihyperlipidemic activity: A review. J Drug Invent Today 2018;10:257-9.
2. Jijith US, Jayakumari S. *In vitro* pharmacological screening methods for anti-inflammatory agents. Asian J Pharm Clin Res Drug 2018;11:1-3.
3. Mishra R, Karmaskar SM, Bhagwat AM. Preliminary dose dependent study on anti-hyperlipidemic activity of *Hibiscus rosa sinensis* Linn leaves on Triton WR 1339 induced hyperlipidemic mice model. Asian J Pharm Clin Res 2011;4 Suppl 2:100-2.
4. Siedel J, Hägele EO, Ziegenhorn J, Wahlefeld AW. Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. Clin Chem 1983;29:1075-80.
5. Sikarwar MS, Patil MB. Antihyperlipidemic activity of *Salacia chinensis* root extracts in triton-induced and atherogenic diet-induced hyperlipidemic rats. Indian J Pharmacol 2012;44:88-92.
6. Narkhede K, Mahajan A, Patil SD. Evaluation of antihyperlipidemic and antiatherosclerotic potential of rimonabant in experimental animals. Int J Pharm Pharm Sci 2013;5:666-70.
7. Ferruzza S, Rossi C, Scarino ML, Sambuy Y. A protocol for differentiation of human intestinal caco-2 cells in asymmetric serum-containing medium. Toxicol *In Vitro* 2012;26:1252-5.
8. Hussein O, Ismail A, Idris OF. Evaluation of serum lipid profile level and gamma-glutamyltransferase activity as a biomarker for coronary artery disease in Sudanese patients. J Appl Pharm Sci 2017;7:16-22.
9. Nigam PK. Serum lipid profile: Fasting or non-fasting? Indian J Clin Biochem 2011;26:96-7.
10. Gaddikeri K, Bhorgonde DD. Estimation of serum lipid profile patterns as a diagnostic marker in oral cancer and precancer. Asian Pac J Health Sci 2016;3:59-62.
11. Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 1998;97:1129-35.
12. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. Science 2001;292:1160-4.
13. Clayton RB, Bloch K. The biological conversion of lanosterol to cholesterol. J Biol Chem 1956;218:319-25.
14. Bloch K, Rittenberg D. The biological formation of cholesterol from acetic acid. J Biol Chem 1942;143:297-8.
15. Dietschy JM, McGarry JD. Limitations of acetate as a substrate for measuring cholesterol synthesis in liver. J Biol Chem 1974;249:52-8.
16. Rodríguez-Sureda V, Peinado-Onsurbe J. A procedure for measuring triacylglyceride and cholesterol content using a small amount of tissue. Anal Biochem 2005;343:277-82.

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