INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online at www.ijpcbs.com

Research Article

CD40 LIGAND MIMETIC MOLECULES ACTIVATE B- CELL PROLIFERATION AND IMMUNOGLOBULIN ISOTYPE SWITCHING

Vemula Vani[1*], Sanjay Prasad[2] and Muthukumaran Sivanandham[3]

[1]Department of Microbiology, M.S. Ramaiah College of Arts, Science and Commerce, Karnataka, Bangalore- 560054, India.
[2]Department of Inorganic and Physical chemistry, Indian Institute of Science, Karnataka, Bangalore- 560012, India.
[3]Department of Biotechnology, Sri Venkateswara College of Engineering, Sriperumbudur-602105, Tamil Nadu, India.

ABSTRACT

Interaction of CD40 ligand (CD40L) with CD40 receptor is one of the most immunologically important interactions that involve in the regulation of T cell dependent B cell proliferation, differentiation and antibody production. Mutation in CD40L is associated with several diseases. Therefore, interfering CD40L interaction with CD40 may have important therapeutic applications. Using the three dimensional structure of CD40-CD40L complex, three small CD40L mimetic molecules have been designed using computational techniques. Out of these three molecules, two molecules viz. ammonium benzoyltrimethyl chloride and N-benzhydrylbenzamide were synthesized and characterized. The third molecule, 3-(Dimethylamino) propiophenone hydrochloride was purchased from a chemical supplier. These three mimetic molecules were tested for their potential to activate B cell proliferation and immunoglobulin isotype switching in the presence of Mycobacterial antigen, PPD. It was found that these molecules at their optimal concentrations have shown to proliferate B cells through the help provided by activated T cells. Moreover, these molecules have switched the IgM isotype to IgG isotype antibody to PPD. Hence, these three molecules can be considered as ideal candidates for further development of novel immunotherapeutic agents.

Keywords: CD40L, Immunoglobulin isotype switching, mimetic molecules, B cells.

INTRODUCTION

The CD40 receptor is a type I membrane protein that belongs to the nerve growth factor gene family. The CD40 Ligand (CD40L), also known as T-B activating molecule, TNF-related activation protein or gp 39, is a 33 kDa type II membrane glycoprotein expressed on the surface of activated CD4+ T cells [1-6]. CD40 receptor is expressed on several cell types like B cells, Dendritic cells, etc. Binding of CD40L to its receptor CD40 on B cells leads to several effects, including B- cell proliferation, prevention of B cell apoptosis resulting in the establishment of immunological memory, germinal center formation, B cell differentiation, immunoglobulin production and immunoglobulin class switching [7-11]. Blocking the interaction between CD40 and CD40L leads to several adverse effects like lack of T cell dependent B cell proliferation and antibody production. CD40L mutation is also found in several diseased conditions like hyper IgM syndrome, arthritis, Hodgkin's lymphoma, hypogammaglobulinemia, and viral infections [12-21]. Therefore, providing CD40L could be a strategy in treating these disease conditions. But use of peptides as drug faces problems of drug administration and delivery. In addition, peptides are unstable and their production is not economical. Use of small molecules as a potent drug has been increasing due to the difficulties in peptide synthesis. Small molecules

are found to exert powerful effects on the functions of macromolecules that comprise living systems making it a useful research tool and a pharmacological agent. Small molecules contribute in unprecedented ways to the understanding and betterment of human health [22]. Hence small molecules that mimic CD40L may prove to be therapeutically effective in treating CD40L deficiency.

Recently, computational methods have been used to discover novel ligands. A collection of small molecules capable of perturbing any disease- related biological pathway is screened using computational methods and this leads to the identification of therapeutic targets [23]. Using these computational methods, many small molecules have been developed for more than 30 targets[24]. These small molecules include the inhibitors of aldose reductase[25], CDK4[26], matriptase[27], Bcl- 2[28], adenovirus protease[29], tyrosine kinase 2[30] and synthetic agonists of cytokine receptors[31, 32].

Using computational methods and the structural information of CD40- CD40L complex, three small molecules that mimic the active site of CD40L were designed in this current study[33]. Out of these three molecules, two molecules viz. ammonium benzoyl trimethylchloride and N-benzhydrylbenzamide were synthesized in the laboratory using organic synthesis methods[34,35]. The third molecule, 3- (Dimethylamino) propiophenone hydrochloride was purchased from Sigma Aldrich, USA. These three compounds were analyzed for their potential to activate B cell proliferation and to switch the immunoglobulin isotype.

MATERIALS AND METHODS

Preparation of CD40L mimetic molecules

Ammonium benzoyltrimethyl chloride (ABTC)

This compound was synthesized by benzoylation of trimethylamine at room temperature. 2ml of trimethylamine (2.8 ml, 0.03 mol) (Sigma Aldrich, USA) and 4ml of benzoylchloride (3.4 ml, 0.03 mol) (Sigma Aldrich, USA) were added in a small conical flask. It was vigorously shaken for 5-10 minutes until the odor of benzoylchloride disappeared. The flask was left overnight for crystal growth. Solid crystal derivative was filtered. It was recrystallized with ethanol (Hyman, England).

N-Benzhydrylbenzamide (NBB)

The N- benzhydrylbenzamide was synthesiszed in three steps. In step1, Phthalimide (Sigma Aldrich, USA) was subjected to N- alkylation to get Diphenyl methyl phthalimide. In step2, Diphenyl methyl phthalimide was subjected to Gabriel synthesis to obtain 1, 1- diphenyl methanamine. In the final step, Schotten-Baumann benzoylation of 1, 1, - diphenyl methanamine was carried out to produce N-benzhydrylbenzamide. The procedure of this synthesis is as follows.

Anhydrous potassium carbonate (76g, 0.55 mol) and phthalimide (147 g, 1 mol) (Sigma Aldrich, USA) were ground together in a glass mortar and treated with bromo diphenyl methane (247.13g, 1 mol) (Sigma Aldrich, USA). The mixture was refluxed at 190°C for 3 hours. Excess bromo diphenyl methane was removed by steam distillation. Diphenyl Phthalimide crystals were collected from the reactants at the end of steam distillation, by filtration using Buchner funnel and washed with water and then with 60% ethanol. Pure diphenyl methyl phthalimide was recrystallized in glacial acetic acid (SD fine chemicals, India) and suspended in hydrazine hydrate (25 ml, 0.5 mol) (Sigma Aldrich, USA). The white gelatinous precipitate formed was decomposed with excess hydrochloric acid. Phthalyl hydrazide precipitate formed was separated by suction filtration and the filtrate was concentrated by distillation on a rotary evaporator. The liberated diphenyl methylamine was extracted with ether, dried with potassium hydroxide and distilled. The pure diphenyl methylamine was suspended in sodium hydroxide and treated with benzoyl chloride for 5- 10 minutes. The formed N-benzhydrylbenzamide was filtered and recrystallized with ethanol.

After purification, these purified compounds were characterized by chemical tests, FT- IR and NMR spectroscopy.

3-(Dimethylamino) propiophenone hydrochloride (3-DPH)

This compound was purchased from Sigma Aldrich, USA. The molecular formula of this compound is $C_{11}H_{15}NO.HCl$. It is a white crystalline powder with M. P 152- 155°C. Its Molecular weight is 213.71 and the purity of compound is 99%.

Stock preparation

A stock with 10mM concentration was prepared for each compound. ABTC and 3-DPH were dissolved in ethanol. NBB was dissolved in ethyl acetate (SD fine chemicals, India). The solvents used had no effect on proliferation of B cells.

Working standard

The working standard concentrations of 5µM, 25µM, 50µM, 100 µM and 10mM were prepared in ethanol for ABTC and 3-DPH and in ethyl acetate for NBB.

Purified protein derivative

Purified Protein Derivative of BCG (PPD) was used as antigen to check the potency of the compounds to stimulate B cells to secrete immunoglobin isotypes. PPD was obtained from MONTAUX test kit (Radiant Parentrals Pvt Ltd, Vaghodia) with activity equivalent to 10 tuberculin units.

Sample collection and processing

Samples for this experiment came from the other team of the laboratory studying the humoral immune respone of the healthy volunteers to PPD Purified protein derivative of BCG) antigen (Manickam et al). Twenty milliliter of blood was collected in EDTA tubes from 20 healthy volunteers via Lions Club Blood Bank, Coimbatore. All volunteers are aged between 25- 28 years. All volunteers were enrolled in the study after getting their consent. From these 20 samples analyzed for humoral immune response, the five samples that showed higher IgM and lower IgG were selected for studying the immunoglobulin isotype switching activity of CD40L mimetic molecules.

The blood was allowed to stand at room temperature for 1 hour and centrifuged at 2500rpm for 10 minutes to collect the plasma. The plasma was diluted (1:2) with Hank's Balanced Salt Solution (HBSS) (HiMedia, India) and was overlaid on a high density Lymphocyte separation medium (Ficoll-Hypaque) (density 1.077 g/L) (PIA Laboratory, Austria) in the ratio of 3:1. It was centrifuged at 1800rpm for 30 minutes. The white puffy layer was collected, and to it thrice the volume of HBSS was added, and centrifuged at 2500rpm for 10 minutes to remove the contaminating ficoll. The cells were washed with RPMI-1640 (HiMedia, India) at 1000rpm for 10 minutes. The cells were then transferred to a T75 tissue culture flask and incubated for one hour at 37° C in CO_2 incubator (Shel Labs, USA). All monocytes were adhered to the flask and only lymphocytes remain suspended in the medium. The non- adherent cells were collected and stored at -20° C.

Nylon wool separation of B cells

The lymphocytes were separated into T and B cells using nylon wool. The nylon wool was washed thoroughly by boiling it in distilled water for 10 minutes. The washing was repeated 6 times and was dried at 37° C for 2-3 days. 1.4gm of Nylon wool was packed in a 20ml syringe up to 1.4ml mark and wrapped in aluminum foil and autoclaved. The nylon wool was then teased in sterile PBS until the strands get separated. Teased wool was then packed onto a 10ml syringe up to 6ml mark and equilibrated at 37° C for 1 hour. The column was now washed with prewarmed RPMI medium and the cells were loaded (1×10^5 cells/ml) into the column. The column was incubated at 37° C for 45 minutes. The non adherent cells were collected by flushing the column with 20ml of RPMI medium. The adherent cells were collected by plunging with cold saline. The cell fractions were centrifuged at 1300 rpm for 10 minutes. The supernatant was discarded and the cells were suspended in 1 ml of fresh medium. The viability of cells in each fraction was determined by tryphan blue staining. Enumeration of T and B cells was done with the T-cell and B-cell lineage markers using flow cytometry.

Flow cytometry

The CD3 and CD19 monoclonal antibodies conjugated with fluorochrome PE-Cy5 (Invitrogen, USA) and phycoerythrin (Invitrogen, USA) respectively were used to stain T cells and B cells. The antibody–stained cells were analyzed using flow cytometry (Beckman Coulter, USA).

B- cell proliferation assay

The B cell proliferation in the presence of small molecules that mimic CD40L binding site (ABTC, NBB and 3-DPH) was assayed. 10µl of $2x10^6$ cells/ ml of B cells were cultured in the presence of 1 µl of IL- 4 (Sigma, USA) and 1µl of varying concentrations of the compound (5µM, 25µM, 50µM, 100 µM and 10mM) in 150µl of RPMI-1640 medium supplemented with L- glutamine, 10% Fetal Bovine Serum (FBS) and antibiotic solution (1% Penicillin/ Streptomycin) in a 96-well U bottomed plate (Tarsons, India) and incubated in a CO_2 incubator for 5 days. On day 5, the extent of proliferation was determined by MTT Assay. For this assay, 100 µl of 3(4,5-dimethly thiazol-2-yl)2,5-diphenyltetrazolium Bromide(MTT) (50mg/ml) (Sigma-Aldrich, USA) was added to the culture and incubated at 37° C for 4 hours in the CO_2 incubator. The formazon crystals formed were dissolved in 100µl of acidified isopropanol (SD fine Chemicals, India) and the colour of formazan was measured at 600 nm. The test was carried out in triplicates to confirm the efficacy of the compound.

The following controls were used in the B cell proliferation assay. PPD activated T cells expressing the CD40L were made nonviable by sudden freezing and thawing and used in the positive control. T cells were activated by culturing $2x10^5$ cells/ml of PBMC in the presence of 10 µl of PPD (10 TU) for 5 days in 5 ml of RPMI- 1640 supplemented with glutamine and antibiotic solution in a T25 tissue culture

flask. After 5 days, 10µl of $2x10^6$ cells/ ml of activated T cells were taken and made nonviable by sudden freezing and thawing. These nonviable T cells were cultured with 10µl of $2x10^6$ cells/ ml of B cells in the presence of 1 µl of IL-4 for 5 days in 150 µl of RPMI- 1640 in a U-bottomed 96 well plate. On day 5, MTT labeling was done to measure the extent of Proliferation. B cells alone cultured in the presence of IL- 4 forms a negative control. For this control, 10 µl of $2x10^6$ cells/ ml of B cells were cultured in the presence of 1 µl of IL- 4 in 150 µl of RPMI- 1640 for 5 days. The extent of proliferation on day 5 was determined by MTT assay. This gives the background proliferation of B cells without the compound.

Assay to measure the antibody isotype switching

An important property of CD40L and CD40R interaction is to bring antibody isotype switching. As the synthesized small molecules mimic CD40L binding site, the ability of ABTC, NBB and 3- DPH to activate immunoglobulin isotype switching was tested by quantifying the amount of IgM and IgG produced by B cells against PPD. The quantification of IgG or IgM immunoglobulin against PPD was carried out by ELISA technique.

The B cell culture supernatant without the compound was used as a negative control whereas the culture supernatant of B cells in the presence of PPD activated nonviable T cells was taken as positive control. The culture supernatants of B cells in the presence of IL- 4 and varying concentrations (5µM, 25µM, 50µM, 100 µM and 10mM) of ABTC, NBB and 3-DPH were tested in the ELISA for IgG and IgM against PPD. Culture supernatants were collected after 24 hours of incubation. The supernatants diluted 1: 25 with PBS were used to measure IgG and IgM against PPD.

ELISA to determine IgG and IgM

The plates were coated with 50µl of 10TU PPD diluted with PBS (pH 7.4) (25µl/ml) and incubated overnight at 4^o C. After the incubation, the plates were washed 6 times with 300 µl of PBS + 0.50% Tween 20 and then blocked with 300 µl of 5% skimmed milk for 1 hour at 37 ^{o} C. Subsequently, the plates were washed 6 times and 100 µl of the sample (B cells culture supernatant diluted 1:25 with 1% BSA in PBS) was added and incubated overnight at 4^oC. The assay was carried out in triplicates.

For quantifying the concentration of IgG in the B cell culture supernatants, the above plates were washed with PBS + 0.5% Tween 20 and incubated with 100 µl of alkaline phosphatase conjugated goat anti- human IgG (Genei Pvt. Ltd., Bangalore, India) at 1: 1000 dilution for 1½ hours at room temperature. The plates were again washed 6 times and 100 µl of substrate (1 mg/ ml), para- Nitro Phenyl Phosphate (pNPP) (Genei Pvt. Ltd., Bangalore, India) was added to develop colour. Substrate buffer for PNPP is combination of ethanolamine and $MgSO_4$. The reaction was arrested after 20 minutes of incubation by adding 100 µl of 3N NaOH solution per well. The yellow colour was measured at 405nm in a microplate reader (Bio-RaD, USA).

Similarly, for quantifying the concentration of IgM in the B cell culture supernatants, after incubating with sample (B cell culture supernatants), the plates were washed and incubated with 100 µl of horseradish peroxidase conjugated goat anti- human IgM (Genei Pvt. Ltd., Bangalore, India) at 1: 1000 dilution for 1½ hours at room temperature. The plates were again washed 6 times and 100 µl of substrate (1 mg/ ml), $3,3^1,5,5^1$ Tetrmethyl Benzidine (TMB) (Genei Pvt. Ltd., Bangalore, India) was added to develop colour. Substrate buffer for TMB is citrate buffer. The reaction was arrested after 20 minutes of incubation by adding 100 µl of 3M H_2SO_4 solution per well. The yellow colour was measured at 405nm in a microplate reader (Bio-RaD, USA).

Statistical method

Student's t test was performed to find the significance in difference between controls and tests. ANOVA was performed to determine the variance among the triplicates in B cell proliferation assay and assay for antibody isotype switching. ANOVA was performed using online statistical software available at http://www.physics.csbsju.edu/stats/anova.html. Results with p values less than 0.05 is considered as statistically significant.

RESULTS AND DISCUSSION

B- cell proliferation assay

The effect of ABTC, NBB and 3- DPH to proliferate B cells was determined using MTT assay. B cell proliferation with the above molecules was compared against the positive control in which B cells were cultured with PPD activated nonviable T cells that provide natural CD40L for the proliferation of B cells. The culture of B cells alone forms the negative control.

As seen in Figure 1, B cell proliferation was found to be the highest at 10mM concentration for ABTC, with the OD value of 2.25. The positive control showed an OD value of 0.173 **±** 0.005 whereas the negative control showed an OD

value of 0.07 ± 0.02. When compared with the positive control that contains nonviable T cells, there is a 13 fold increase in B cell proliferation with ABTC at 10mM concentration. The p value of the triplicate was found to be 0.017 indicating that the ABTC aided proliferation is statistically significant.

As seen in Figure 1, the NBB was found to produce the maximum B cell proliferation with the OD value of 2.161 at 100 µM concentration. At this concentration, there is a 5 fold increase in B- cell proliferation due to NBB as compared to the positive control that produced an OD of 0.173 ± 0.05. The negative control produced the OD of 0.07±0.02. The p value of the triplicate was found to be 0.004, indicating that the proliferation of NBB is statistically significant.

The 3- DPH was also found to induce B cell proliferation. The maximum B cell proliferation effect of 3- DPH was found to be at the concentration of 50 µM with an OD value of 2.362. There was a 10 fold increase in B cell proliferation with 3-DPH at 50 µM concentration as compared to the positive control. The p value of the triplicate was calculated as 0.035, indicating that the 3- DPH proliferation results are statistically significant.

Antibody isotype switching studies

As mentioned in methodology, from the 20 volunteer samples analyzed for their humoral response to PPD, the five samples showing higher concentration of IgM and lower concentration of IgG in the plasma were selected to study the immunoglobulin isotype switching activity of ABTC, NBB and 3- DPH against PPD.

The concentrations of IgM and IgG of the B cell culture supernatant in the presence and absence of ABTC were measured against PPD. IgM concentration in the presence of optimum concentration of ABTC in culture supernatant was found to decrease (OD value 0.074) when compared to the negative control (OD value 0.091) which contains no compound. There is an increase in IgG concentration in culture supernatant came from the B cell culture treated with ABTC. At 10mM concentration of ABTC, there was a 3 fold (OD value 0.194) increase in IgG concentration as compared to the negative control (OD value 0.065) (Figure 2). The p value of the triplicate was calculated as 0.014, which shows that the ABTC induces a statistically significant increse in the secretion of IgG from B cells. This result clearly shows that the ABTC-aided immunoglobulin isotype switching from IgM to IgG is taken place.

The IgM and IgG concentrations against PPD in the presence and absence of NBB were measured in the B cell culture supernatants and the results are presented in Figure 2. The IgM concentration in the presence of optimum concentration of NBB was found to decrease (OD value 0.077) when compared with the negative control (OD value 0.091). There was an increase in IgG concentartion in culture supernatant derived from B cells treated with NBB. The increase in IgG concentartion was found to be the highest at 100 µM concentration of NBB. There was a 3 fold (OD value 0.184) increase in IgG concentartion at 100 µM concentration of NBB compared to the negative control (OD value 0.065). The p value of the triplicate was calculated as 0.026, thus suggesting that NBB induces statistically significant increase in the secretion of IgG.

Similarly, the IgM and IgG concentrations against PPD were measured in B cell culture kept in the presence and absence of 3- DPH and the results are presented in Figure 2. The IgM concentration in the presence of optimum concentration of 3-DPH was found to decrease (OD value 0.087) compared to the negative control (OD value 0.091). There is an increase in IgG concentration of supernatant in the presence of 3-DPH. At the concentration of 50 µM of 3- DPH, IgG concentration was increased to 3.2 folds (OD value 0.207) as compared to control (OD value 0.065). The increase is a statistically significant increase in igG concentration with 3-DPH, with the p value 0.009.

CONCLUSION

The interaction between CD40 and its ligand contributes to regulation of B cell proliferation, immunoglobulin production, immunoglobulin class switching, germinal center formation and development of B cell memory[7-11]. This study indicates that the CD40L mimetic small molecules may enhance the IgG immune response to antigens. In addition, these molecules can be used in diseases that have immunoglobulin switching problems such as hyper IgM syndrome, arthritis, Hodgkin's lymphoma and hypogammaglobulinemia.

Using the structural information of CD40/CD40L complex, three small molecules that mimic CD40L binding site have been designed[33]. Among these three molecules, two molecules viz. Ammonium benzoyltrimethyl chloride and N- benzhydrylbenzamide were syhesized by organic methods and they had been purified and chararcterized by FTIR and NMR spectroscopy[34,35]. The third molecule, 3-(Dimethylamino) propiophenone hydrochloride was purchased from a chemical supplier.

All these three compounds were tested for their biological activity. As CD40L can induce B cell

proliferation and is involved in the control of immunoglobulin (Ig) class switching, these three CD40L mimetic molecules (ABTC, NBB and 3-DPH) were assessed for their B cell proliferation and antibody isotype switching activities. All the three mimetics were found to activate proliferation of B cells more effectively than the controls. In addition, ABTC, NBB and 3- DPH have also shown immunoglobulin isotype switching from IgM to IgG. Hence, these three molecules can be considered as ideal candidates for further development of novel immunotherapeutic agents.

Figure 1 : Effect of CD40L Mimetic Molecules on B Cell Proliferation

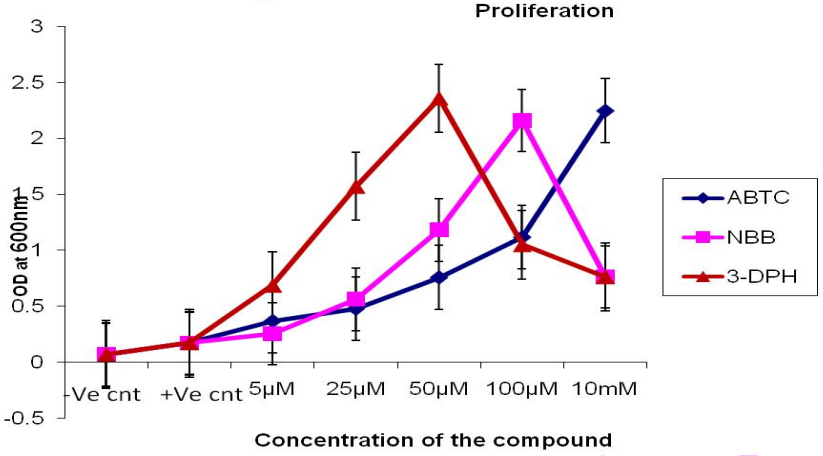

Fig. 1: This graph shows the B cell proliferation with the ABTC/ NBB/ 3- DPH. When B cells were cultured with 5µM - 10mM concentrations of the compound, a higher proliferation was obtained with 10mM concentration for ABTC, with 100µM concentration for NBB and with 50 µM concentration for 3-DPH when compared to control. The values included are the mean of triplets. The bars represent the SE. The mean OD values of the positive control and negative controls were found to be 0.173±0.05 and 0.07±0.02

Figure 2: Effect of CD40L mimetic molecules on Antibody Isotype Switching

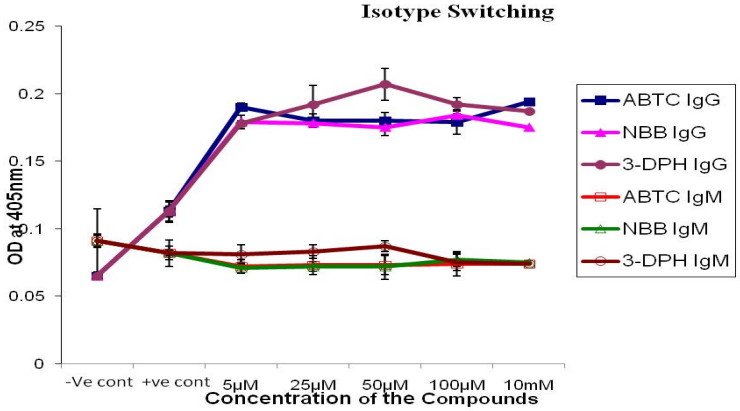

Fig. 2: The graph shows the IgM type and IgG type anti-PPD antibody concentrations of B cell culture supernatants in the presence and absence of the ABTC, NBB and 3- DPH. The IgM concentration was found to decrease by 1.5 fold at the optimum concentrations of ABTC, NBB and 3- DPH compared to the control which contains no compound (OD value 0.091) . IgG concentration was found to increase by about 3 fold in the presence of optimum concentrations of ABTC, NBB and 3- DPH against negative control (OD value 0.065) . The optimum concentrations of ABTC, NBB and 3- DPH were found to be 10mM, 100 µM and 50 µM respectively. In positive control the IgM concentration was 0.082±0.0002 and the IgG concentration was 0.113±0.029.

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