Characterization and validation of fragment antigen-binding (Fab) antibody-based immunoassay for deoxymiroestrol, a potent phytoestrogen from Pueraria candollei

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Abstract

Deoxymiroestrol is the most potent phytoestrogen in chromenes group that has been found in Pueraria candollei, Thai name known as Kwao Krua Khao. Several studies reported estrogenic activity of P. candollei in order to using as hormone replacement therapy for postmenopausal women. Previously, specific determination of deoxymiroestrol content by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody (pAb) have been reported. However, production of pAb has limitation and variability from different batches. Therefore, in this study, we established quantitative method for determination of deoxymiroestrol using fragment antigen-binding (Fab) antibody-based immunoassay. The developed immunoassay has specificity to deoxymiroestrol with a calibration range of 15.6-1000 ng mL-1. Precision including intra-assay and inter-assay are 1.48-7.11 and 0.58-9.31%, respectively. Accuracy of the assay showed in recovery between 99.77-101.61% when spike deoxymiroestrol standard into the samples. The limit of detection (LOD) is 30.80 ng mL-1. Comparation antibody-based immunoassay for determination of deoxymiroestrol using Fab with pAb was represented consistency (R2 = 0.9807) when analysis roots bark of Pueraria candollei from difference areas. Therefore, this development assay can apply to determine deoxymiroestrol content in the plant samples.

Introduction

Tuberous root of *Pueraria candollei* (Thai name Kwao Krua Khao) is an ingredient in Thai traditional medicines and many health supplements for using as hormone replacement therapy in women with menopause symptoms. A systematic review of *P. candollei* for menopausal treatment in clinical trials revealed that efficacy and safety remain inconclusive as a result of non-standardized products with potent phytoestrogen compounds like deoxymiroestrol and miroestrol.[1] Quantitative analysis of deoxymiroestrol and miroestrol especially in *P. candollei* is high recommended by the National Consumer Affairs Center of Japan (NCAC) for the avoidance of undesirable physiological effects. Phytoestrogen chemical contents in *P. candollei* need to be declared in raw material and products distributed in the global market to guarantee the safety and therapeutic effects of this plant. The estrogenic makers of*P. candollei* are deoxymiroestrol, miroestrol, isomiroestrol and methylisomiroestrol[2]; however, deoxymiroestrol is the most potent phytoestrogen activity. The quantitative analysis of deoxymiroestrol faces many challenges.

The high-performance liquid chromatography coupled UV-Vis detector (HPLC) produced too low sensitivity

(LOD = 200 ng $mL^{-1}$for miroestrol and 780 ng mL^{-1} for deoxymiroestrol) and selectivity to apply for samples with low content of miroestrol and deoxymiroestrol.[3] The LC-MS-MS[4] solved the problem in which the sensitivity of miroestrol and isomiroestrol analysis was enhanced (LOD = 4.17 and 0.84 ng mL^{-1} respectively), but its complex procedure and equipment suffer the routine analysis with the cost. In addition, the main drawback of these techniques is that toxic solvents are involved.

Immunoassay-based method using antibody like an indirect competitive enzyme-linked immunosorbent assay (icELISA) for determination of bioactive compounds in medicinal plant has drawn much attention due to its advantages of being sensitive, rapid, simple to operate and cost-effective method. Interesting, the method is environmentally friendly because no organic solvents are needed.[5] Immunoassay can be developed using various forms of antibodies such as IgG, Fab, scFv, etc. However, production of mAb using hybridoma culture showed cross reactivity to both miroestrol and deoxymiroestrol (MD-mAb), therefore, immunoassay was developed to determine total deoxymiroestrol and miroestrol in plant samples and products.[6] We reported icELISA method using polyclonal antibody (pAb) for specific determination of deoxymiroestrol.[7] The method was sensitive and reliable. Unfortunately, pAb has variability among different batches production. For scFv, this type of antibody was generated as unnatural structure therefore it was not stable for long-term usage.

The advantages of recombinant Fab for analytical method were less post-translational modification, single gene template and less environmental dependent variation. Fab production in *Escherichiacoli* used short time to produce antibody about 1-3 days. Purification of Fab in *E. coli* is not more steps compare with insect larvae using immobilized metal affinity chromatography, gel filtration, and anion exchange chromatography to purify Fab.[8]

Presently, no recombinant antibody for determination deoxymiroestrol. Different between hybridoma produced MD-mAb and *E. coli* expressed Fab may affect the characteristics of antibody. *E. coli* derived Fab cause analytical performance and might be used instead of anti-deoxymiroestrol pAb.

Currently, we aim to produce functional Fab antibody in the engineered*E. coli* SHuffle® stain, and then characterized the binding properties in comparison with the parental mAb. The analytical performances of Fab based icELISA were characterized for sensitivity, specificity, precision, recovery, and the correlation with validated methods. The Fab based icELISA is applicable for the determination of deoxymiroestrol in the samples of *P. candollei* . Besides, the stability of the Fab was studied. Overall, the *E. coli* derived Fab proved as the practical and sustainable source of icELISA for deoxymiroestrol. Therefore, this study aims at developed icELISA method for determination of deoxmiroestrol using Fab antibody. The developed Fab-based ELISA method was validated for its sensitivity, precision and accuracy. We also reported the development of a Fab-based icELISA method to determine deoxymiroestrol in *P. candollei* plant samples. Modification of this immunoassay using Fab can improve stability of recombinant antibody and apply as sustainable resource of antibody.

Materials and Methods

Chemicals and reagents

Authentic deoxymiroestrol, miroestrol, isomiroestrol, and methylisomiroestrol were isolated from *P. candollei* and identified by ^{1}H-NMR spectroscopy, as previously described. [9,10] Yeast extract powder and tryptone for terrific broth (TB) medium were purchased from Himedia (Mumbai, India). Peroxidase-conjugated anti-mouse IgG (Fab specific) antibody was purchased from Sigma-Aldrich (MO, USA). Complete His-tag purification resin was purchased from Roche (Mannheim, Germany). The*E. coli* SHuffle$^{(r)}$ T7 strain occupying the pET21b (+) vector/MD-Fab gene (cassette No. 1) was from the previous study.[11] All reagents were analytical grade.

Expression of recombinant MD-Fab

The *E. coli* SHuffle$^{(r)}$ T7 occupied recombinant pET21b (+) vector/MD-Fab was cultured in 2 L Erlenmeyer flask with 500 mL terrific broth (TB) medium supplemented with 100 mg mL^{-1} ampicillin. The culture was

performed at 30 degC, 150 rpm shaking. After the growth *E. coli* cell reached to log phase (OD_{660} 0.6-0.8), IPTG solution was added for a final concentration of 1 mM. The expression was induced under temperature 25 degC, 150 rpm shaking for 18 hours. Then the cell was collected via centrifugation at 4 degC, 8000 rpm, 5 min. Twenty milliliters of lysis buffer (50 mM Tris–HCl pH 8, 1 mM EDTA, and 10% (v/v) glycerol) was added to wash the cell. After well suspending, centrifugation was performed again. After that, the cell was incubated for 30 minutes with lysis buffer containing lysozyme (0.5 mg mL^{-1}) for destruction of *E. coli* cell wall. Then, the digested mixture was adjusted for final concentration of 0.5 M NaCl and 1% Triton X-100, which use for lysing cell. The cell pellet was additionally incubated in an icebox for 30 minutes, and ultrasonication was applied to disrupt cells. After centrifugation at 4 degC, 13000 rpm for 30 minutes, the soluble protein was collected as the crude protein of purification of Fab.

Purification MD-Fab

The C-terminus of the heavy chain (V_H-C_H1) was tagged with the hexahistidine (HIS_6). Thus, Ni-affinity was used to purify the Fab from the soluble fraction. Setting Ni-affinity column (2 mL) had been initially equilibrated with 10 mM imidazole in 50 mM Tris-HCl buffer pH 7.4, and then soluble protein was applied to the column. Then, unbound proteins were washed with 10 mM imidazole (10 mL) and 20 mM imidazole (10 mL) in 50 mM Tris-HCl buffer pH 7.4, respectively. Finally, the elution solution (200 mM imidazole in the same buffer, 10 mL) was applied. The eluted proteins were collected. The column was additional cleaned with 0.5 M imidazole in 50 mM Tris-HCl buffer pH 7.4 (5 mL) and then re-equilibrated with 10 mM imidazole in 50 mM Tris-HCl buffer pH 7.4 (10 mL). The Ni-affinity column was kept in 20% ethanol at 4 degC until the next usage. The eluted fraction was dialyzed in 20 mM Tris-HCl, pH 8 at 4 degC for three times, which the imidazole would be removed. Then, the concentration of eluted protein was determined using the Bradford reagent, where the BSA was used as the reference protein. The presence and size of Fab in the eluted fraction was revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing condition. The purified Fab was characterized for binding properties using indirect and indirect competitive ELISA.

Indirect ELISA using MD-Fab

The procedure of indirect ELISA was used to reveal the binding activity of the Fab toward the conjugated form of miroestrol (Mi-HSA). The useful Fab for development of icELISA has to recognize both conjugated form and free form of miroestrol. The conjugation reaction of Mi-HSA was explained in the previous research.[12] The first step of indirect ELISA was the coating of Mi-HSA on the surface of microtiter plates, which a hundred microliters of Mi-HSA (4 μg mL^{-1} in 50 mM sodium carbonate buffer pH 9.6) was added in each well. Then, the plate was incubated for 1 hour. The non-attached Mi-HSA was washed with 0.05% Tween 20 in phosphate-buffered saline (pH 7.0; 0.01 M) (T-PBS). Each well was then filled with 300 μL of 5% skim milk solution in PBS and incubated for 1 hour. The skim milk protein would clock free surface from non-specific adsorption in consequence procedure. After the skim milk was removed, 50 μL of 5% ethanol and an equal volume of purified Fab in T-PBS at varied concentrations were added and incubated for 1 hour. The unattached Fab was discarded, and the Mi-HSA bound antibodies were reacted with peroxidase-conjugated anti-Fab antibody (100 μL, diluted 1: 1000 with T-PBS) was added to each well. After reaction for 1 hour, the plate was washed, and 100 μL of substrate solution [sodium citrate buffer (pH 4.0; 100 mM) containing 0.003% H_2O_2 and 0.3 mg mL^{-1} ABTS] were added. After incubation at 37 °C for 20 minutes, the absorbance was measured at 405 nm by a microplate reader (Biochrom, EZ Read 400, UK). Absorbance at 405 nm was plotted against concentrations of MD-Fab. The optimal concentration of Fab would be selected for further analysis.

Indirect competitive ELISA using MD-Fab and method validation

Indirect competitive ELISA (icELISA) was developed based on the competitive binding between free and immobilized miroestrol conjugate toward the Fab. When the concentration of immobilized Mi-HSA was fixed, the icELISA was correlated to the concentration of authentic miroestrol. This icELISA using MD-Fab was validated and applied for the determination of deoxymiroestrol content in *P. candollei* samples. In the

procedure, all steps were performed as indirect noncompetitive ELISAs until the step of skim milk treatment. The binding reaction was performed in each well after the various concentrations of deoxymiroestrol prepared in 5% ethanol, and 50 µL MD-Fab solution (3.40 µg mL^{-1}) were well mixed. In the well of control, 50 µL 5% ethanol and 50 µL of the same MD-Fab concentration. The inhibitory curve of deoxymiroestrol against MD-Fab was plotted betweenA/A_0 and the logarithmic scale of compound concentration. The absorbance of the ELISA signal of the well with the sample was defined as A , and A_0 was the absorbance of the control well.

The binding specificity was evaluated by the experiment of cross-reactivity. The cross-reactivity was calculated from the half-maximal inhibitory concentration (IC_{50}) of deoxymiroestrol and investigated compounds against the reactivity of the MD-Fab binding to Mi-HSA:

$$\text{CR } (\%) = \frac{\text{IC50 of deoxymiroestrol}}{\text{IC50 of the investigated compound}} \times 100$$

Linearity is the range of concentration that shows a linear relationship with icELISA absorbance. The concentration that provides 10% inhibition ($A/A_0 = 0.9$) was set as the limit of detection (LOD).[13] The variation of the developed method was calculated as the relative standard deviation (RSD). Seven levels of deoxymiroestrol concentrations within the linearity range were investigated. Intra- and inter-assay precision were evaluated by RSD between wells (n =3) within a plate and RSD between plates (n =3), respectively.

The experiment of recovery was designed the assess the accuracy of the Fab based icELISA. The ethanol extract of *P. candollei* root was used as the sample for the experiment. Then, the authentic deoxymiroestrol solution was spiked into the extracts for the final concentrations of 75, 150, and 300 ng mL^{-1}. The developed icELISA determined the deoxymiroestrol contents of spiked and non-spiked extracts. The accuracy was judged from the percentage of recovery:

$$\text{Recovery } (\%) = \frac{(\text{deoxymiroestrol in the spiked sample} \quad \text{deoxymiroestrol in the non}-\text{spiked sample})}{\text{spiked amount of deoxymiroestrol}} \times 100$$

The validated method was applied to determine the deoxymiroestrol content in different *P. candollei* samples. The obtained results were compared with icELISA using pAb being specific to deoxymiroestrol.[7]

Stability of MD-Fab

Recombinant antibody was modified from mAb, the structure of Fab antibody differs from the parent antibody which might affect the conformation and functional of Fab during storage. Consequently, stability of MD-Fab was examined for long-term usage. In order to determine stability of MD-Fab, the Fab was stored in the three preservative conditions including T-PBS (F-1), 1% (w/v) BSA in T-PBS (F-2) and 1% (w/v) BSA plus 0.02% (w/v) NaN_3 in T-PBS (F-3). Two temperature points were studied (4 °C and -20 °C). The reactivity of the stored Fab against deoxymiroestrol was evaluated at the time interval after storage (1, 2, 3, 4, 6 weeks, 2, 4, and 6 months). The binding activity was evaluated using indirect competitive ELISA. The experiments were performed in triplicate.

Plant materials and extraction

The tuberous roots of *Pueraria candollei* samples from the different areas were collected, dried, and ground into powder. The specimens were deposited at the Herbarium, Faculty of Pharmaceutical Sciences, Khon Kaen University (NI-PSKKU 121-126). The homogenous samples of every sample (200 mg) were extracted with absolute ethanol 1 mL for four-times using sonicator 15 minutes and centrifuge 8000 rpm for 10 min. The extracted solution was evaporated until dry at room temperature. The remaining residues were dissolved with 1 mL ethanol and then diluted as the working concentration in 5% ethanol in purified water for icELISA. Each sample was done in triplicate.[12]

Results and Discussion

According to our previous report of expression of actively soluble MD-Fab antibody in SHuffle® T7 *E. coli* strain, Fab protein obtained from pET21b (+) vector/MD-Fab gene cassette no. 1 can display in the active form and has higher specificity against deoxymiroestrol than the parental monoclonal antibody.[11] The

functional Fab was expressed and fold in the *E. coli* cell. Using SHuffle® T7*E. coli* as expression system, the active form of Fab antibody was achieved in the cytoplasm of cell without refolding process which provides useful method when compared with conventional *E. coli*strain. Therefore, this cassette was selected for the expression of the MD-Fab antibody in order to apply for the development of the immunoassay method.

After expression and purification, MD-Fab from the affinity column was calculated to 2.55 mg per *E. coli* culturing media one liter. As shown in Figure S1, two bands of proteins were observed after purification. The bigger one corresponds to the Fab with theoretical size of 450 amino acid is 50 kDa protein and the other one being smaller with 18 kDa approximately. The major protein was observed with molecular weight of 50 kDa that assembled of VH-CH1 and VL-CL the same as previously reported.[11] The smaller protein in that band might be VH-CH1 which His-tag protein can attach with Ni-affinity column and this band of protein did not form disulfide bond with VL-CL. The uncompleted assembly of Fab might cause by the conditions during culturing of SHuffle® T7 *E. coli* . However, VH-CH1 fragment did not affect immunoassay method because anti-Fab specific region was used as secondary antibody and small amount of VH-CH1 was formed. Therefore, purified recombinant MD-Fab antibody can be used for icELISA method development.

The reactivity of purify Fab was examined with direct ELISA, MD-Fab was diluted to 0.09-48.57 µg mL^{-1} in T-PBS and reacted with 5% ethanol. The results presented in Figure 1a. The concentration of MD-Fab which exhibits the optimal absorbance and inhibition with free antigen was observed. Appropriated concentration for studying indirect competitive ELISA was 3.40 µg mL^{-1}.

For the cross reactivity with substances in chromenes group, MD-Fab has specific against deoxymiroestrol with less cross reactivity to miroestrol 43.55% compare with the original MD-mAb (72.87%). Besides, cross reactivities against other chromenes, isomiroestrol and methylisomiroestrol show 31.87 and 9.84 %, respectively (Table S1). It might cause by the different forms of antibody. The Fab antibody have only one antigen binding site which could affect its binding characteristic and conformation during icELISA assay. A previous study also reported the different in cross reactivity of Fab and mAb against paclitaxel-related compounds. [14]

The results indicated that MD-Fab has higher specific against deoxymiroestrol and bind miroestrol less than MD-mAb mother template. This result was similar to our previous study.[11] Thus, determination of deoxymiroestrol by icELISA using MD-Fab antibody was developed.

The icELISA against various concentrations of deoxymiroestrol was performed. The linearity of calibration curve ranged from 15.62 to 1000.00 ng mL^{-1} (Figure 1b). The limit of detection (LOD) is 30.80 ng mL^{-1}. The results indicated that Fab-based icELISA has narrow range when compare with pAb-based icELISA from our previous study[7], which was 0.73 to 3000.00 ng mL^{-1}, even if the sensitivity was slightly inferior. The icELISA using MD-Fab was approximately 26 times more sensitive than HPLC method (LOD of HPLC method = 780 ng mL^{-1}).[3]

For method validation, precision was determined by intra- and inter-assay of deoxymiroestrol (n=3). Intra- and inter-assay precisions are displayed as % RSD in Table 1 with the range of 0.58-9.31% and 1.48-7.11%, respectively. The accuracy of the assay was validated by spiking known amounts of standard deoxymiroestrol into *P. candollei* tuberous root extracts and then amount of deoxymiroestrol was determined. Accuracy of the assay presents as recovery percentage of spiked deoxymiroestrol in the range of 99.77-101.61% (Table 2). The results from method validation assays confirmed that the developed recombinant Fab-based icELISA was sensitive and accurate for determination of deoxymiroestrol. It suggested that other cross reactivity substances were not affected to the developed method. The developed icELISA using MD-Fab was applied for quantitative determination of deoxymiroestrol content in *P. candollei* samples.

The developed icELISA using recombinant antibody MD-Fab was performed to determination of deoxymiroestrol in plant samples to compare with the result from pAb specific deoxymiroestrol.[7] As shown in Table 3, our results displayed the consistency of deoxymiroestrol content in the samples from tuberous root of *P. candollei* obtained from both MD-Fab and pAb (R^2 = 0.9807). Therefore, icELISA using MD-Fab can be used for determination of deoxymiroestrol contents in *P. candollei* raw materials.

In order to determine stability of MD-Fab as recombinant antibody-based icELISA, activity of MD-Fab at various conditions was performed.[15] Fab antibody may have limited stability under denaturing conditions including temperature and stabilizing solution. Therefore, the storage conditions were design from practical using antibody for ELISA. Our study shows that MD-Fab can stable during keeping for 24 weeks in almost all conditions except in 1% BSA in T-PBS (F-2), 4 ºC (Figure 2). It can be noted that the value of IC_{50} obtained from 1% BSA in T-PBS, 4 ºC condition (F-2) at week 24 higher than other conditions. Among all conditions for study of MD-Fab stability, 1% BSA and 0.02% NaN_3 in T-PBS (F3), kept in -20 ºC was the optimal condition to maintain MD-Fab activity. This information base on our knowledge to keep the recombinant antibody stable. NaN_3 is widely used for microbial preservative and 1% BSA was added for reducing surface tension and non-specific absorption between container and antibody as well as reducing proteolysis of antibodies.[16]

In this study, functional MD-Fab antibody was directly expressed in the soluble form using *E. coli* Shuffle T7 strain and additional refolding is not needed. The analytical performed validated prove the reliability for quantification of deoxymiroestrol by icELISA. The developed icELISA using MD-Fab is appropriate and sustainable method for quality control amount of deoxymiroestrol from *P. candollei* plant samples. The method might be appliable for other samples such as supplement products or biological fluids in clinical studies. In addition, Fab-based antibody is stable, cost effective and sustainable source when compared with exist polyclonal antibody.

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Conflict of interest statement

The authors declare that they have no conflict of interests.

Author Contributions

Worapol Sae-Foo: Formal analysis, Investigation, Methodology, Writing original draft, Visualization. Wipawee Juengsanguanpornsuk: Investigation. Supaluk Krittanai: Investigation, Resources. Gorawit Yusakul: Validation, Writing - review & editing. Tharita Kitisripanya: Investigation, Methodology. Seiichi Sakamoto: Investigation. Waraporn Putalun: Conceptualization, Methodology, Validation, Writing - review & editing.

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Figure captions

Figure 1 (a) Indirect ELISA reactivity of MD-Fab at varied concentrations against 5% ethanol (b) Indirect competitive ELISA exhibiting the reactivity of Fab against deoxymiroestrol

Figure 2 IC_{50} of MD-Fab against deoxymiroestrol at various conditions for 24 weeks. **F-1** : T-PBS,**F-2** : 1% BSA in T-PBS and **F-3** : 1% BSA plus 0.02% NaN_3 in T-PBS. The solid lines were kept in 4 ^{o}C and the dot lines were kept in -20 ^{o}C.

Table 1 Precision of ELISA by MD-Fab

Deoxymiroestrol (ng mL^{-1})	Deoxymiroestrol (ng mL^{-1})	% Relative stan
		Inter-assay (n=
1000.00	1000.00	5.16
500.00	500.00	4.91
250.00	250.00	2.23
125.00	125.00	0.58
62.50	62.50	3.68
31.25	31.25	3.04
15.62	15.62	9.31
[a] % RSD = (standard deviation (S.D.)/mean)×100	[a] % RSD = (standard deviation (S.D.)/mean)×100	[a] % RSD = (sta

Table 2 Recovery of deoxymiroestrol as determined by MD-Fab ELISA

Spiked level (ng mL^{-1})	Measure amount (ng mL^{-1}) [a]	Recovery (%)
0	168.94 ± 13.89	-
75	243.77 ± 24.03	99.77
150	318.70 ± 22.09	99.84
300	473.78 ± 44.97	101.61
[a] The data present in mean ± SD (n=3)	[a] The data present in mean ± SD (n=3)	[a] The data present in mean ± SD (n

Table 3 Deoxymiroestrol contents in samples determined by icELISAs

	Deoxymiroestrol contents (µg g^{-1} dry wt.) [a]	Deoxymiroestrol contents (µg g^{-1} dry wt.) [a]
Samples	pAb based icELISA	Fab based icELISA
P. candollei 1 (Bangkok)	38.86 ± 3.32	42.73 ± 2.86
P. candollei 2 (Bangkok)	44.20 ± 1.13	44.71 ± 5.19
P. candollei 3 (Bangkok)	5.81 ± 0.87	6.89 ± 1.36
P. candollei 4 (Bangkok)	29.09 ± 1.56	34.4 ± 3.99
P. candollei 5 (Loei)	9.69 ± 0.65	6.87 ± 0.36
P. candollei 6 (Nakhon Nayok)	11.87 ± 1.11	12.59 ± 1.76
a The data present in mean ± SD (n=3)	a The data present in mean ± SD (n=3)	a The data present in mean ± SD (n=3)

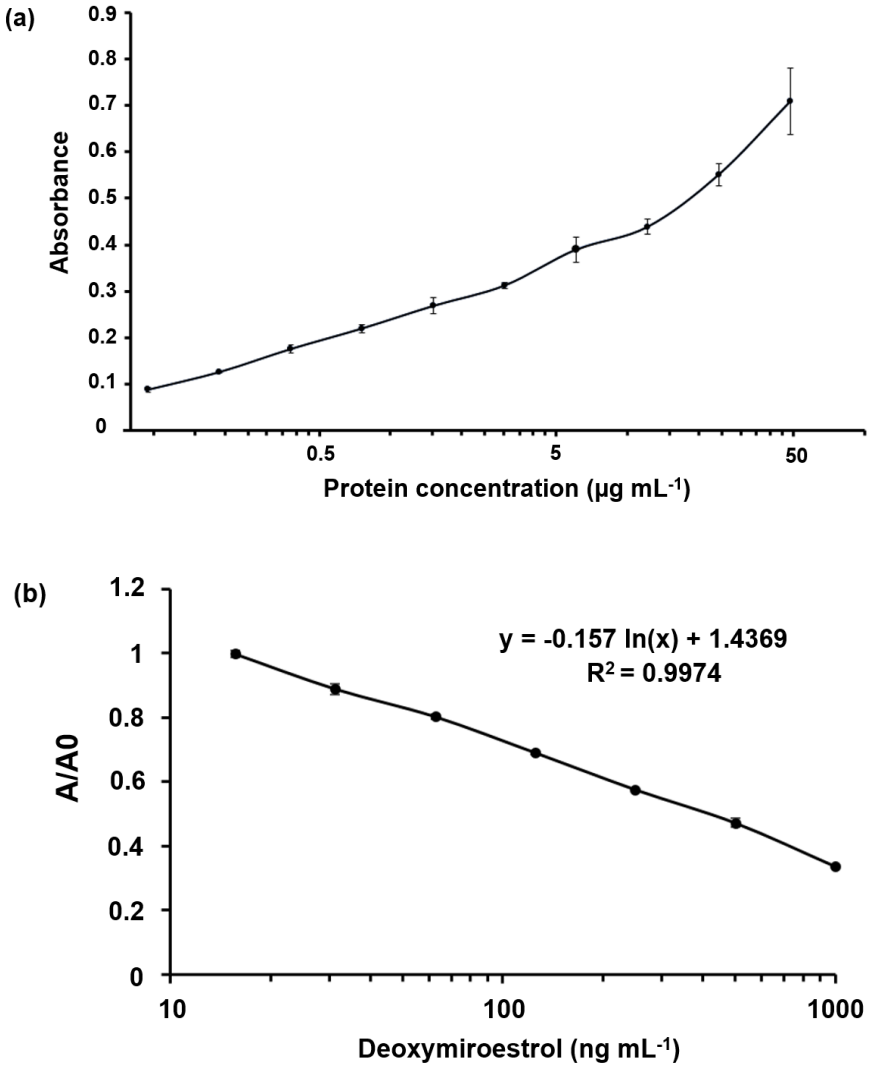

Figure 1 (a) Indirect ELISA reactivity of MD-Fab at varied concentrations against Mi-HSA **(b)** Indirect

competitive ELISA exhibiting the reactivity of Fab against deoxymiroestrol

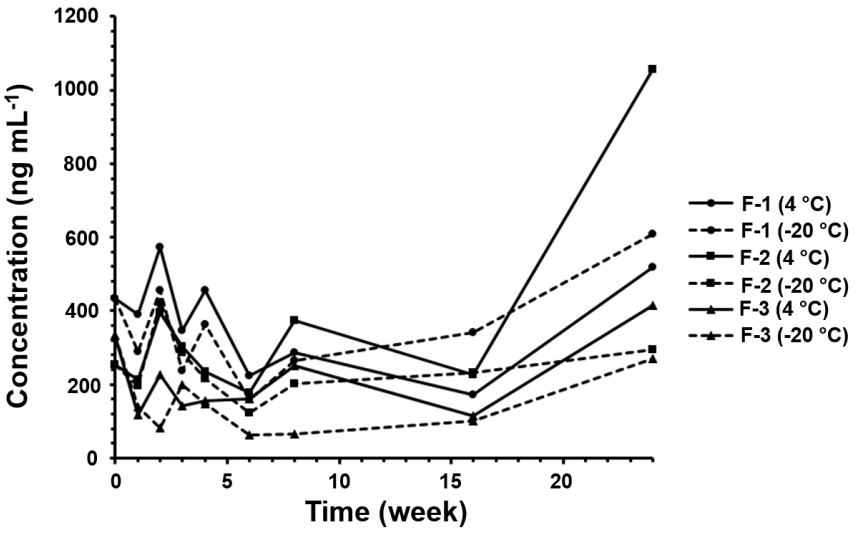

Figure 2 IC_{50} of MD-Fab against deoxymiroestrol at various conditions for 24 weeks. **F-1** : T-PBS,**F-2** : 1% BSA in T-PBS and **F-3** : 1% BSA plus 0.02% NaN_3 in T-PBS. The solid lines were kept in 4 ^{o}C and the dot lines were kept in -20 ^{o}C.