CHAPTER III

MATERIALS AND METHODS

1. Chemicals and Equipment

Authentic artemisinin was obtained from the Plant Biochemistry and Plant Physiobiochemistry Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.

[1-^{3}H(N)] Farnesyl diphosphate triammonium salt, (specific activity = 16.1 Ci/ mmol, 0.5 mCi/ml) was purchased from Perkin Eler Life Sciences Inc., Boston.

3-(N-morpholino)-propanesulfonic acid (Mops), ascorbic acid, glycerol, bovine serum albumin (BSA), β-nicotinamine dinucleotide phosphate (reduced form), tetrasodium salt (NADPH), *dl*-dithiothreitol (DTT) were purchased from Sigma Chemical Company, St Loius, Mo., USA. Dihydrogen orthophosphate ($Na_2HPO_4.12H_2O$) and sodium dihydrogen ortophosphate ($NaH_2PO_4.H_2O$), magnesium chloride ($MgCl_2$), and sodium molipdate (MoO_4) were obtained from Merck, Damstadt, Germany. Coomassie brilliant G-250 (for protein analysis) was purchased from Bio-Rad Laboratories, Richomo, Ca., USA. Liquid nitrogen and nitrogen gas were purchased from Thailand Industrial Gases, Samutprakan, Thailand.

Organic solvents were all reagent grade or better from LAB-SCAN Analytical Scinces, Dublin, Ireland and Merck, Damstadt, Germany. Water was triple deionized.

Thin-layer chromatography plates, Silica gel POLYGRAM® SIL G/UV254 was purchased from MACHEREY-NAGEL GmbH& Co. KG, Germany.

Detection of Radioactive was performed by using TLC Linear analyzer LB 284/285, Berthold, Germany.

Densitometric technique was performed by using CAMAG TLC SCANNER 3, samples were applied on TLC plate by LINOMATE 5 and the data were analyzed by win CATS-Plana Chromatography Manager program Version : 1.4.2.8121 (product ID 144w007) from CAMAG, Switzerland.

Polymerase Chain Reaction was performed by Mastercycler from eppendorf, Germany.

Sonicator was performed by Soniprep 150 from Sanyo, Japan.

Agarose gel visualization was demonstrated by Gel Doc™ XR 170-8170, Bio-Rad, Italy.

2. The Effect of Gamma Irradiation on *A. annua* Plantlets and Plant Transfer

2.1 Plant material

The seeds of *Artemisia annua* were obtained from Vietnam. The seeds were surface-sterilized in 10 % Chlorox®(0.35 % sodium hypochlorite) for 20 min and in 5 % Chlorox® for 10 min. After being washed three times with sterilized water, the seeds were propagated in MS medium (Table 4) containing 3 % sugar and 0.8 % agar. The germinated seedling cultures were kept in 40 $\mu mol.m^{-2}.s^{-1}$of fluorescence light for 2 months. The plantlets were then subcultured five times before being subjected to γ-ray irradiation. Practically, the shoots were removed from the plantlets,

Table 4 MS basal media (Murashige and Skoog, 1962)

Macronutrients	mg/l
NH_4NO_3	1,650
KNO_3	1,900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micronutrients	mg/l
H_3BO_3	6.20
$MnSO_4.2H_2O$	6.90
$ZnSO_4.H_20$	6.14
KI	0.83
$Na_2MoO_4.H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron	mg/l
Sodium EDTA	37.25
$FeSO_4.7H_2O$	27.85
Organic components	mg/l
Glycine	2.0
Nicothinic acid	0.5
Pyridoxine	0.5
Thiamin	0.1
Sucrose	30 g/l
pH	5.5

cut to the sized of 0.5 cm long on to the same MS medium. Two hundred obtained shoots were irradiated with γ-ray 650 of Cobalt-60 (dose rate 856 rad.min^{-1}) in three doses as 0-10 Gray (Gy) cordially coorperated by the Office of Atomic Energy for Peace (OAEP). The exposed shoots were then transferred to the fresh MS medium with the same ingredients. The shoots were subcultured every six weeks for three times. In the forth subculturing, the plants were propagated to many clones for selecting heat tolerance strains and then transferred to the bottle culture which has filter on the cover. The heat tolerance strains were selected by growth the plant in the medium under the temperature as 25 °C and 30 °C for six weeks and then picked up the serviced clone. After the selection, the leaves were collected from the plants and extracted with hexane. The hexane extracts were then concentrated before being tested for antimalarial activity. The plant which has high antimalarial activity under temperature at 30 °C and the leaves of 200 clones in each irradiation dose were selected for determination of artemisinin and test antimalarial activity again.

A. annua variants were grown with plant tissue culture technique at the Plant Biochemistry and Plant Biochemistry Laboratory, National Center for Genetic Engineering and Biotechnology, Thailand.

2.2 *In vitro-ex vitro* transfer

After 6 weeks of *in vitro* culture, some irradiated plantlets with normal appearance were transferred into acclimation conditions in 16 oz glass bottle. The bottle was contained 50 ml vermiculite that absorbed 50 ml MS medium without sucrose. One membrane filter (Milli-Seal, Millipore, Japan; pore size: 0.5 μm) was attached over a hole (area: 0.8 cm^2) on the plastic cap for air exchange according to

the method described by Kozai et al. (1986). For the acclimation step, all the cultures were grown at 25±1 °C under 40 μmol.m^{-2}.s^{-1} photosynthetic photon flux density provided by cool-white fluorescence lamps. Then, the plants were transferred to 4 ins pot with sterile soil at 25±1 °C under 40 μmol.m^{-2}.s^{-1} photosynthetic photon flux density provided by cool-white fluorescence lamps. After 15 days, the plants were transferred to green house of Chulalongkorn University, Bangkok, and some of them were transferred to grow in the open field (30-38/20-29°C, day/night, air temperature) at Kanchanaburi Province, Thailand. Plantlets were harvested and determined for their artemisinin content.

3. Cloning and Expression of Amorpha-4,11-Diene Synthase Gene in *Artemisia annua*

3.1 Plant material

The seeds of *Artemisia annua* were obtained from Vietnam. The seeds were surface-sterilized in 10 % Chlorox®(0.35 % sodium hypochlorite active ingredient) for 20 min and in 5 % Chlorox® for 10 min. After being washed three times with sterilized water, the seeds were propagated in MS medium (Table 4) containing 3 % sugar and 0.8 % agar. The germinated seedling cultures were kept under 40 μmol.m^{-2}.s^{-1}of fluorescence light for 2 months. The plantlets were then subcultured five times before being subjected to RNA extraction.

3.2 Total RNA isolation and cDNA synthesis

Total RNA was isolated from the young leaves of *A. annua* by using Trizol method (Wang *et al.*, 2006). Fresh leaves were weighed for 200 mg and ground with

1 ml Trizol in a cool mortar. The mixure was transferred into 1.5 ml microfuge tube and incubated at room temperature for 5 min. Chloroform was then added into the mixture (0.2 ml per 1 ml Trizol), shaked immediately for 15 second and future incubated at room temperature for 10 minutes. The mixture was centrifuged at 10,000 rpm at 4 °C for 15 min. Then the supernatant part was transferred into a new 1.5 ml microfuge tube and 0.5 ml isopropanol was added and inverted mixed. The mixture was incubated at room temperature for 10 min and centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was discarded and the RNA pellet was washed by 1 ml 70 % ethanol. The RNA pellet was then centrifuged at 10,000 rpm at 4 °C for 5 min. The supernatant was discarded and the pellet was dried by holding microfuge tube at room temperature. The RNA pellet was resuspended in 10 µl DEPC-H_2O and incubated at room temperature until the RNA was dissolved. The concentration of RNA was determined by spectrophotometry under the wavelength of 260 nm.

3.3 DNA amplification by reverse transcriptase-polymerase chain reaction (RT-PCR)

The obtained total RNA was amplified as cDNA pool of *ads* gene by using *Revers*-IT™ 1STStrad synthesis kit from ABgene, USA. The reaction mixture contained 1 µl RNA template solution and 1 µl 1st stand primer (Oligo dT 500 ng/µl) and was made to the volume of 13 µl with sterile water. The mixture was heated at 70 °C for 5 min and kept on ice for 5 min. The reaction mixture was then mixed with 4 µl of 5x first strand synthesis buffer, 2 µl of dNTP (5mM of each dATP, dTTP, dGTP and dCTP) and 1 µl reverse-IT Reverstranscriptase Blend, followed by invert mixed and spinned down. The reaction mixture was incubated at 47°C for 30 min and inactivated the reverse transcriptase by incubating the reaction at 75 °C for 10 min.

The total cDNA from this reaction was kept at -20 °C for full time use. For synthesizing the *ads* gene from the cDNA library, the PCR amplification of the *ads* gene was obtained by using programmable DNA thermal cycler. The reaction was performed in 50 µl total volume with the final concentration of each component: one time of one step buffer (15mM $MgCl_2$), dNTP (0.2 mM of each dATP, dTTP, dGTP and dCTP), 0.02 U of Taq DNA polymerase, 0.2 µM of each *ads* gene specific primer (a forword primer *ads*1 : 5'CG GGATCC ATGTCACTTACAGAAG and revers primer *ads*2 : 5'CGA CTCGAGTCATATACTCATAGGATAAA, which included the restriction sites (underlined) for *BamH* I and *Xho* I. The reaction was then added with 1 µl of the cDNA template from above preparation. The PCR mixture was started for 5 min denature at 94 °C and 30 times for 30 s at 94 °C, 1 min annealling at 56 °C and 2 min extension at 72 °C (Table 5). The PCR product was determined by using agarose gel electrophoresis.

Table 5 Thermal cycler conditions for PCR amplification of DNA fragments of *ads* gene

PCR condition	Temperature (°C)	Times
Initial denaturation	94	5 min
Three step cycling		
Denaturing	94	30 s
Annealing	56	1 min s
Extension	72	2 min
Number of Cycles	30	
Final Extension	72	4 min

3.4 Digestion with restriction endonuclease

Plasmid and *ads* gene fragments digestion were performed by using the buffer provided with the enzyme under the conditions recommended by the manufacturer (Takara Product, Japan). Digested plasmid and *ads* gene fragments were purified by using Gene clean kit (Takara Product, Japan), the digested products were separated by agarose gel electrophoresis.

Each band of the plasmid and *ads* gene fragments was cut form the agarose gel and transfer into microtube. Three volume of NaI buffer was added in one volume of the gel and incubated at 55 °C for 10 min (or until the gel slice has completely dissolved). Then the glass milk was added for 5 µl and vortex. The microtube was kept on ice and slowly invert mixed for each 1 min for 10 min. The tube was centrifuged at 6,000 rpm for 1 min at 4°C, discarded supernatant. The pellet was resuspended with 200 µl washing buffer on ice and centrifuged at 6,000 rpm for 1 min at 4 °C. The supernatant was discarded. This washing step was performed 2 times. The pellet was resuspended with sterilized distill water and then incubated at 55 °C for 5 min. The solution was centrifuged at 15,000 rpm for 10 min at room temperature. The DNA was in the solution and rechecked the DNA purity by agarose gel electrophoresis.

3.5 Ligation of plasmid and insert DNA

The estimation of DNA concentration was performed by agarose gel electrophoresis with standard molecular weight. In practice, either 1:1 or 1:3 concentration ratio of vector and insert DNA was used. The ligation kit was perchased from Takara Product (Japan).

3.6 Medium (Sambrooke, 1989)

SOC medium

20 % (w/v) Bacto-tryptone

5 % (w/v) Bacto-yeast extract

0.5 % (w/v) NaCl

The solution was added with 10 ml of 250 mM KCl, adjusted to pH 7.0 with NaOH, and then adjusted to the volume of 11 ml. The mixture was autoclaved, 5 ml of sterile 2 M $MgCl_2$ was added (19 g $MgCl_2$ in 100 ml H_2O).

LB medium

1.0 % (w/v) Tryptone

0.5 % (w/v) Yeast extract

1.0 % NaCl

Adjusted to pH 7.0 with 1 M NaOH

3.7 Plasmid transformation into *E. coli*

One hundred microliters of competent cells were transferred into a reaction tube and then it was mixed with 2 µl of ligation reaction mixture. The tube was swirled, incubated for 30 min on ice, immersed in water bath for 90 second at 37 °C, and then transferred immediately on ice for 5 min. Two microliters of SOC medium (prewarm at 37 °C) were then added into the tube, and shook at 200 rpm for 1 hr. The cells were inoculated onto LB agar plates contained 30 µg/ml kanamycin for over night at 37 °C. The colonies were selected and detected for gene insertion by PCR, using specific primers for *ads* gene.

3.8 Alkaline extraction (Birnboim and Doly, 1979)

The culture medium was centrifuged at 12,000 rpm for 5 min at 4 °C and the supernatant was discarded. The pellet part was added with 100 µl Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0). The solution was vortexed and kept on ice for 5 min. Solution II (0.2 N NaOH, 1 % SDS) was then added 200 µl, invert

mixed and kept on ice 5 min. Finally, Solution III (5 M potassium acetate solution) was added for 150 µl, invert mixed and kept on ice for 5 min. The solution was then centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was transferred to new tube. For the new tube, the 300 µl supernatant was invert mixed with 700 µl 99 % ethanol. The mixture was kept on ice for 5 min. Then the mixture was centrifuged at 14,000 rpm for 5 min at 4°C, the supernatant was discarded. The pellet was rinsed with 1 ml of 70 % ethanol and centrifuged at 14,000 rpm for 5 min at 4 °C. The pellet was redissolved in 20 µl of 10 fold dilutions TE buffer and 20 µg/ml RNase then kept at -20 °C.

3.9 Preparation of soluble and insoluble proteins from *E. coli*

Single colony of the transformed *E. coli* was grown in LB medium containing 2.5 µg/ml kanamycin at 37°C for 3 h and to obtain OD_{600} of 0.6. Then isopropyl β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.5 mM. The culture was shook in incubator shook at 200 rpm, 38 °C for 4 h. After adding IPTG (0 min), the culture was collected in each 30 min. The cell suspension was centrifuged at 5,000 rpm for 5 min at 4°C and the supernatant was discarded. Then, the pellet was resuspended in 10 % Triton X-100 and protein was extracted by using ultrasonic probe (Soniprep 150, Sanyo) for 30 second two times on ice. The suspension was centrifuged at 14,000 rpm for 20 min at 4 °C. Both the supernatant and pellet parts were determined protein expression by SDS-PAGE.

3.10 Electrophoresis

3.10.1 Agarose gel electrophoresis

Agarose gels were prepared for separation of DNA with the size of 0.5-25 kb. The gel concentration of 0.5 % - 1 % agarose was used for small DNA fragments. One gram of agarose gel was dissolved with 100 ml 1x TAE buffer using microwave. The obtained gel solution was poured in a agarose gel apparatus. DNA sample was mixed with 5x loading buffer (Fermentus) and pipetted into the well of the gel. The loading gel apparatus was connected to a power supply, and run at 100 volts.

50x TAE buffer

Tris-base	240	g
Glacial acetic acid	57.1	ml
0.5 M EDTA	100	ml

Adjusted to pH 8.0, and adjusted the volume to 1 L

3.10.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis on 12 % polyacrylamide gel was performed according to the method of Laemmli (Laemmli. 1970). SDS-PAGE was used to determine the overexpression of protein from the transformed *E. coli.* It was performed by Bio-Rad Mini Protean II Apparatus on a Mini-Slab gel (70 x 80 x 0.75 mm). The separating gel contained 12 % polyacrylamide (prepared from a stock solution of 30 % w/v acrylamide (Biol-Rad).

Five microliters of sample were added with the sample buffer and heated for 5 min at 95°C. The sample preparation was then centrifuged for 1 min at 13,000 rpm to

precipitate the insoluble matters, 10-20 µl of protein sample was loaded into the gel well.

3.10.3 Coomassie blue stain

The gel was immersed into the coomassie blue staining buffer for one hour, then it was destained with the destainig buffer with gently shaking.

Separating gel 12 %

Water	3.4	ml
1.5 M Tris/HCl, pH 8.8	2.5	ml
20 % SDS (w/v)	50	µl
Acrylamide/Bisacrylamide		
(Rotiphorese® gel 30, Roth)	4	ml
10 % Ammonium persulfate		
(Serva, analytical grade)	50	µl
TEMED	5	µl

Stacking gel 4 % gel

Water	3.075	ml
0.5 M Tris/HCl, pH 6.8	1.25	ml
20 % SDS (w/v)	25	µl
Acrylamide/Bisacrylamide	670	µl
10 % Ammonium persulfate	25	µl
TEMED	5	µl

5x Running buffer, pH 8.8 1 L

Tris-Base (Ultraquality, Roth)	15	g
Glycine	72	g

SDS	5	g
Water q.s.	1	L

1x sample buffer 8 ml

Water	4	ml
0.5 M Tris/HCl. pH 6.8	1	ml
Glycerol	800	µl
10 % SDS	1.6	ml
β-Mercaptoethanol (Roth)	400	µl
0.05 % Bromphenol Blue (Sigma)	200	µl

Coomassie Blue stain solution

Glacial acetic acid	100	ml
Methanol	300	ml
Water	600	ml
Coomassie Brilliant Blue G-250 (Roth)	1	g

Destaining solution

Glacial acetic acid	100	ml
Methanol	300	ml
Water	600	ml

3.11 Plant transformation

The shoots of *A. annua* were cultured *in vitro* for 4-6 weeks. *A. tumifeciens* strain LBA4404 harboring the binary vector pBI121/ADS was grown in 2 ml LB medium containing 10 µg/ml kanamycin with shaking at 250 rpm and 28 °C for 2 days. In part of plant transformation system, a sterile syringe needle with *A. tumefeciens* LBA4404 harboring the binary vector pBI121/*ads* from the liquid culture

and then scratched the shoots and stems of *A. annua* for about 1-2 mm deep. The inoculated explants were incubated on MS medium (30 g/l sucrose, 8 g/l agar) at 25 ^{o}C for 2 day under the dark room. After the co-culture period, the explants were transferred to selected medium containing kanamycin and cefotaxime (Claforan®). The selected medium was added with 10 mg/l kanamycin for each step of the transformation process. After being subcultured for one month in the selected medium, the explants were transferred to the second medium contained 300 mg/l cefotaxime and 10 mg/l kanamycin for one month. Then, the explants were transferred to the third medium containing 100 mg/l cefotaxime for another one month. Finally, the explants were transferred to the medium without the antibiotics. For the shoot regeneration, plant hormones were applied in each step of selected medium contained 0.1 mg/l TDZ. The survival explants were transformed to be calli at the scratched wound. When the regenerated shoots became 2-3 cm in length, they were transferred to a hormone-free MS medium for stimulation of roots and shoots elongation for 6 weeks. Subsequently, the shoots were micropropagated and then collected to determine for the presence of pBI121/ADS transformation, the ADS enzyme activity and artemisinin content of the transgenic *A. annua*.

3.12 PCR analysis of transgenic *A. annua*

3.12.1 DNA isolation

Genomic DNA was isolated from the shoots of the regenerated *A. annua*. The fresh shoot was weighted (5 mg) and transferred into 1.5 ml microtube then using DNesy Kit (QIAGEN, USA).

3.12.2 PCR condition

The PCR reaction of CaMV 35s promoter was performed by using Mastercycle (eppendorf). The specific primer including primer S1 (sense) 5'-GCT CCT ACA AAT GCC ATC A-3'and S2 (anti-sense) 5'-GAT AGT GGG ATT GTG CGT CA-3' were synthezied by SIMA Laboratory (Singapore). The reaction was obtained in 50 µl final volume. The final concentration of each component was 1x PCR reaction mix, 8 mM $MgCl_2$ 200 µM dNTP mix (0.1 mM of each dATP, dGTP, dCTP and dTTP), 0.1 µM of sense and anti-sense primers, 1.5 U of *Taq* and 1 µg approximately DNA template. The reaction was mixed and spinned down. The step cycling of a PCR reaction was followed in Table 6. At the end of the reaction, the PCR products were stored at 4 °C. The 10 ml of PCR products were analyzed on 1 % agarose gel.

Table 6 Thermal cycler conditions for PCR amplification of DNA fragments of CaMV 35s promoter

PCR condition	Temperature (°C)	Times
Initial denaturation	95	3 min
Three step cycling		
Denaturing	95	50 s
Annealing	57	42 s
Extension	72	45 s
Number of Cycles	45	
Final Extension	72	4 min

3.13 DNA sequencing reaction

The PCR product was purified by Millipore DNA purification kit (Millipore, USA). The DNA sequencing reaction was used reaction mixture of Genome Lab Tm Dye Terminator Cycle Sequencing for Quick Start Kit (BeckMan Coulter). The reaction was prepared in 0.2 ml thin-wall tube. All reagents should be kept on ice

while preparing the sequencing reaction and should be added in the order as listed below.

ddH_2O (to adjust total volume to 20 µl)	4	µl
DNA template (purified PCR)	6	µl
Primer (1.6 µM in each sense and antisense primers)	2	µl
DTC Quick Star Master Mix	8	µl
Total	20	µl

Thermal cycling program:

96 °C	20 s
50 °C	20 s
60 °C	4 min

for 30 cycles followed by holding at 4 °C.

For removing the exceed reaction mixture, the sequencing reaction was purified by illustaTM AutoSeq G-50 Dye Ter minator Removal Kit (GE Healty Care, Swithzerland). The purified sequencing reaction was sequenced by using the automate CEQTM 8000 Genetic Analysis System (BeckMan Coulter).

4. Artemisinin Analysis by TLC-Densitometric Method

The analysis of artemisinin was performed by TLC-densitometric method described previously (Koobkokkuuad *et al.*, 2007). In practice, the leaves of *A. annua* harvested for 1.5 month after subcutured were dried in a hot air oven. The dried leaves from various samples of *A. annua* variants were ground and extracted under reflux with 10 ml hexane for 4 hours in a 20 x 2.5 cm tube connected with a 15 cm condenser. After cooling and precipitating of the extracted powder, the clear solution of crude extract was spotted directly (20 µl) onto a precoated silica gel

(POLYGRAM^{R} SIL G/UV_{254}, 0.25 mm thinness, Merck, Germany) and developed by the solvent system of hexane:ethylacetate: acetone, 16 : 1 : 1. The TLC plate was then exposed with saturated ammonia at 100 °C for 2 hours for chromophore development of artemisinin. The TLC plate was then scanned using TLC-densitometer (CAMAG TLC SCANNER 3, winCATS-Plana Chromatography Manager program, Version 1.4.2.8121, CAMAG, Switzerland) under wavelength of 313 nm.

5. Preparation of Crude Enzyme Extracts

Fresh leaves (5g) of *A. annua* were quick frozen in liquid nitrogen and ground in pre-cooled mortar. The resulting fine powder was added with 15 ml cold extraction buffer containing 5 mM Mops buffer, pH 7.0, 10 %(v/v) glycerol, 1 mM ascorbic acid, 10 mM $MgCl_2$ and 2 mM DTT. After being stirred for 15 min, the suspension was passed through four layers of cheese-cloth and the filtrate was centrifuged 100,000x*g* at 4°C. The supernatant was then desalted on through a PD-10 column. The filtrate was used as "crude enzyme extract" and kept in –80 °C refrigerator before using.

6. Enzyme Assay for Amorpha-4,11-Diene Synthase

For an enzyme assay of amorpha-4,11-diene synthase, a reaction mixture used contained [1-^{3}H(N)]FDP (100,000 cpm), 5mM Mops buffer, pH 7.0, 10%(v/v) glycerol, 10 mM ascorbic acid, 10 mM $MgCl_2$, 2 mM DTT and 10 mM Na_2MoO_4 in total volume of 70 µl. After being incubated at 30 °C for 30 min, the reaction mixture was extracted with 1 ml hexane for 20 second using vortex. The hexane fraction was then taken and evaporated (Speed Vac®) and spotted onto a TLC plate (POLYGRAM® SIL G/UV_{254}, 0.25 mm thinness). The resulting TLC plate was then developed in a solvent system of hexane:ethylacetate:acetic acid; 25:7:1 and then

scanned to obtain radiochromatograms by TLC-radioscanner (Linear analyzer B284/285; Berthold, Germany).

7. Protein Determination

Various enzyme solutions were determined for their protein content by the method of Bradford (1976). In practice, each enzyme solution was taken 160 µl to be mixed with 40 µl of the commercial available dye reagent concentrate (Bio-Rad protein assay) in a 96-well microtiter plate. The mixtures in the plate were incubated at room temperature for 5 min followed by reading their absorbance which was measured at the wavelength 595 nm using Bio-Rad Model 450 Microplate Reader. A calibration curve of BSA followed its linearity of the relationship between BSA (0-70 µg) and absorbance 595 nm (0-0.8). This calibration curve was used for converting the obtained absorbance values to their protein concentrations.

8. Statistical analysis

All statistical analysis used for estimating the effect of the irradiation on the artemisinin content and enzyme activity of ADS was determined using two way ANOVA (SPSS®), Chicago, IL, USA).