CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

All chemicals used in this study were analytical grades listed as following:

Chemicals	Companies
Absolute Ethanol	Merck
DL-alpha-tocopherol (Vitamine E)	Fluka
L-ascorbic acid	Fisher
2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Fluka
Boric acid	Scharlau
Curcumin	Sigma
Ethidium bromide	Vivantis
Ethyl acetate	J.T.Baker
Ethylene diamine tetra acetate (EDTA)	Fisher
Lamda DNA-Hind III	Amersham
3-morpholinosydnonimine (SIN-1)	Sigma
Naphthylethylenediamine dihydrochloride	Fluka
Reduced β-nicotinamide adenine dinucleotide dinucleotide (NADH)	Sigma
Nitroblue tetrazolium (NBT)	Sigma
3-nitrotyrosine	Sigma
Phenazin methosulfate (PMS)	Sigma
Potassium chloride	Merck
Potassium dihydrogen phosphate	Scharlau
Potassium persulphate	Carlo erba
Rutin	Fluka

Chemicals	Companies
Sodium acetate trihydrate	Fisher
Sodium chloride	Merck
Sodium hydroxide	Scharlau
Sodium nitroprusside (SNP)	AnalaR
Sulphanilamide	Fluka
Tris base	USB
L-tyrosine	Sigma

3.2 Instruments

Instruments	Companies
Automatic micropipette	Gilson
Electronic Analytical Balance	Sartorius
Electrophoresis set	Amersham
Filter paper	Whatman
Freeze drier	Chirst
Freezer	Sanyo
Gel documentation	Bio-Rad
Hot air oven	Memmert
Microcentrifuge	Beckman
pH meter model 654	Metrohm
Soxhlet's apparatus	
UV/VIS Spectrophotometer Jasco model 7800	Jasco
UV/VIS Spectrophotometer Shimadzu model UV-2450	Shimadzu
Ultrasonic Sonicator	Elma
Vortex mixer	Scientific industries
Water bath	Heto

Instruments	Companies
Microplate UV/VIS Spectrophotometer	Beckman Coulter
High Performance Liquid Chromatography (HPLC)	Hewlett packard

3.3 Preparation of medicinal plants in Northern Thailand extract

Six kinds of medicinal plants in Northern Thailand, *Caesalpinia sappan* L., *Leea rubra* Blume ex Spreng., *Syzygium albiflorum* Bahodur & R.C. Guar ST., *Vernomia volkameriforia* Wall.ex DC., *Schleichera oleosa* Merr. and *Holoptelea integrifolia* Planch. were collected from San Pa Thong district, Chiang Mai province, Thailand in May, 2005. The specimens were identified and deposited at the herbarium of Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.

The samples were cut into small pieces, dried in a hot air oven (60^{o}C, 48 hours) and ground. About 500 g of each sample was extracted separately with 95% ethanol using soxhlet's apparatus for 48 hours to obtain ethanol extracts. The extracts were concentrated and dryness by evaporation under vacuum. The dried samples were kept in refrigerator (2-8^{o}C).

3.4 Optimization of *in vitro* antioxidative capacity methods

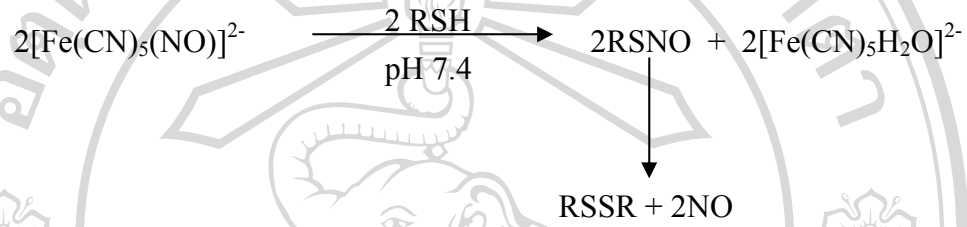
3.4.1 Superoxide anion radical scavenging activity assay

Superoxide anion radicals were generated in a non-enzymatic system using phenazine methosulfate (PMS) and reduced β-nicotinamide adenine dinucleotide dinucleotide (NADH) system by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). The chemicals and reaction mixture were scanned from 200-700 nm spectrophotometrically. The reaction mixture containing 3.5 mM of NADH, 2.5 mM of NBT, 0.5 mM of PMS, and 2.5 mM of EDTA were prepared by dissolving the powders in phosphate buffered saline (PBS) pH 7.4. Then 200 µl of EDTA, NADH, and NBT were mixed and diluted with PBS to the volume of 20 ml (reagent A). The superoxide anion radicals were generated in 250 µl of PBS buffer containing 200 µl of the reagent A, then 25 µl of PMS was finally added to initial the reaction. At 1 minute interval, the absorbance of reaction mixture was observed at 560 nm by UV/VIS Spectrophotometer Jasco model 7800 for 10 minutes. The plot between the absorbance

and reaction time exhibited the best incubation time for superoxide anion radical production from NADH (modified from Yanping, 2004).

3.4.2 Nitric oxide scavenging activity assay

Sodium nitroprusside (SNP) does not liberate NO spontaneously *in vitro*. It required partial reduction (one-electron transfer) by a variety to reducing agents in body tissues. For example, thiols can perform this function:



Scheme 10. Sodium nitroprusside generated nitric oxide by reducing agent (thiols)

It can also be decomposed to NO by light (hν):

Scheme 11. Sodium nitroprusside decomposed to nitric oxide by light (hν)

Sodium nitroprusside is actually a source of nitrosonium ion (NO^+), and thus behaves as a nitrosating electrophilic species (Albert, 2000).

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reagent (1% sulfanilamide and 0.1% naphthyletylenediamine dihydrochloride in 2% H_3PO_4). 6.25 mM of SNP was prepared by dissolving the powder in phosphate buffered saline (PBS) pH 7.4. The reaction containing SNP (0.8 ml, final concentration 5 mM) and PBS (0.2 ml) were incubated at 25°C for 210 minutes. At 30 minutes intervals, the reaction mixture 150 µl of the incubation was removed and diluted with 150 µl of Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyletylenediamine. The absorbance spectrum of maximum wavelength of the reaction was observed at 540 nm by UV/VIS Spectrophotometer Jasco model 7800. The plot between the absorbance and

incubation time exhibited the best incubation time for nitrite production from SNP (modified from Sreejayan and Rao, 1997).

3.5 Optimization of DNA damage protection-induced by Fenton reaction

Plasmid DNA can readily be isolated in this supercoiled form. One approach to detecting both single strand breaks and double strand breaks caused by the direct effect of radical species on DNA, is to analyze their effects on migration properties of small supercoiled DNAs in agarose under the influence of an electric field. The generation of hydroxyl radical ($HO^{\bullet}$) through Fenton reaction from hydrogenperoxide (H_2O_2) required the presence of trace amounts of transition metal ions, especially of Fe, which showed in scheme 2. H_2O_2 is extremely short-lived and reacts rapidly with almost every cellular biomolecules including DNA (Chenyang, 2005). $OH^{\bullet}$ can damage DNA through hydrogen transfer from C-H units of the sugar and addition to all the bases.

3.5.1 Determination of suitable plasmid DNA concentration

Different concentration of plasmid DNA PUC18 (initial from 25-100 ng/µl) in buffer solution was incubated at 37°C for 60 minutes. After incubation, the solutions were applied to 1% agarose gels under a 0.5x TBE buffer system. The electrophoresis was performed at 100 V for 90 minutes. Following electrophoresis, gels were stained with 0.3 µg/ml ethidium bromide for 30 minutes. After washing, the bands were visualized under the UV transilluminator.

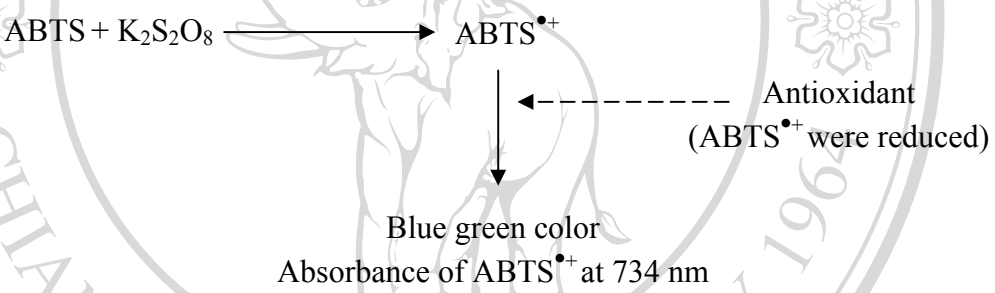
3.5.2 Determination of suitable hydrogenperoxide concentration

The reaction mixture contained 50 ng/µl plasmid DNA PUC18 in buffer, fixed 8 µM of Fe(II) and varying concentrations of H_2O_2. After incubated at 37°C for 60 minutes, the reaction mixtures were applied to 1% agarose gels under a 0.5x TBE buffer system, and electrophoresis was performed at 100 V for 90 minutes. Following electrophoresis, gels were stained with ethidium bromide for 30 minutes. After washing, the bands were visualized under a UV tramsilluminator. The modification of the fluorescence intensity of the bands is due to DNA strand breakage that leads to a decrease in the proportion of supercoiled form (form I) and to an increase in relaxed form produced by a nick in one strand (form II) and linear duplex produced by cleavage of both strands (form III).

3.6 Evaluation of antioxidant activity

3.6.1 ABTS free radical cation decolorization assay

The ABTS, 2,2'-azinobis (3-ethylbenzothaizoline-6-sulfonic acid) diammonium salt radical cation decolorization test a spectrophotometric method widely used for the assessment of antioxidant activity of various substance. The experiments were carried out using an improved ABTS decolorization assay with some adaptation (Re, 1999). It is applicable for both lipophilic and hydrophobic compounds. $ABTS^{\bullet+}$ was generated by oxidation of ABTS with potassium persulfate as shown in the following scheme (Peerajan, 2006)



The capability of scavenge the ABTS free radical was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of negative control} - \text{Absorbance of sample}}{\text{Absorbance of negative control}} \times 100$$

Reagents

ABTS, Potassium persulfate, Standard L-ascorbic acid (vitamin C), and Standard trolox

Procedure

ABTS was dissolved in water to a 7 mM concentration + 2.5 mM Potassium persulfate

⇓

$ABTS^{\bullet+}$ working solution + Samples or Standard solution

⇓

Measured at 734 nm

(Spectrophotometer Shimadzu model UV-2450)

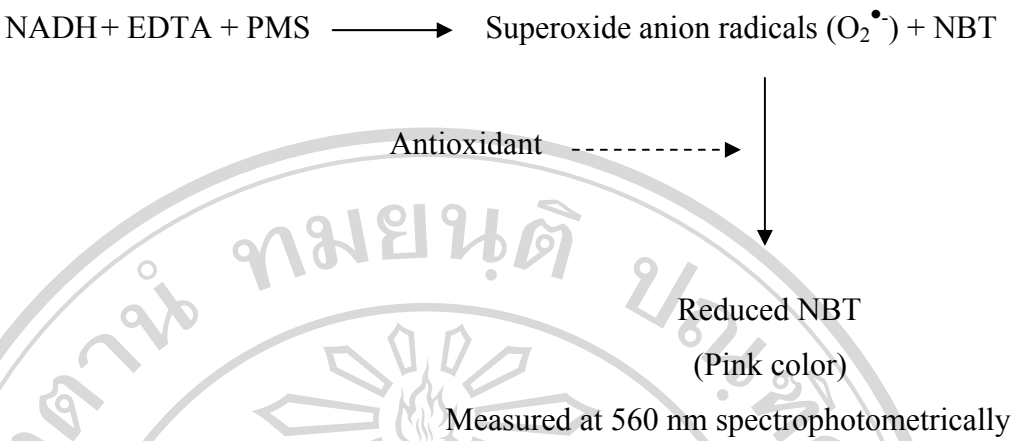
Scheme 12. The protocol of ABTS free radical cation decolorisation assay

3.6.2 Superoxide anion radical scavenging activity assay

Superoxide anion radicals were generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide anion radicals were generated in 250 µl of PBS buffer (pH 7.4), which containing 3.5 mM of NADH, 2.5 mM of NBT, 0.5 mM of PMS, 2.5 mM of EDTA, and samples at different concentrations with initial concentrations were from 10 to 1000 µg/ml were prepared in 96 well plate, comparing to standard L-ascorbic acid and rutin (positive control). PMS was finally added to initial the reaction. After 5 minutes incubation at room temperature, the color reaction between superoxide anion radical and NBT was measured at 560 nm spectrophotometrically. The mixture without sample was used as a control and the mixture without PMS was used as a blank (modified from Yanping, 2004).

The capability of scavenge superoxide anion radical was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of negative control} - \text{Absorbance of sample}}{\text{Absorbance of negative control}} \times 100$$



The 50% effective concentration (EC_{50}) was calculated according to the relationship of concentration and %inhibition.

Reagents

Reduced β-nicotinamide adenine dinucleotide (NADH), Phenazin methosulfate (PMS), Nitroblue tetrazolium (NBT), Ethylene diamine tetra acetate (EDTA), Standard L-ascorbic acid (vitamin C), Standard rutin, Potassium chloride, Potassium dihydrogen phosphate, Sodium acetate trihydrate, and Sodium chloride

Procedure

EDTA, NADH, NBT, and PMS were dissolved in PBS (pH 7.4)

⇓

EDTA, NADH, and NBT were mixed and diluted with PBS to give a reagent A

⇓

Reagent A + PMS + Samples or standard solution

⇓

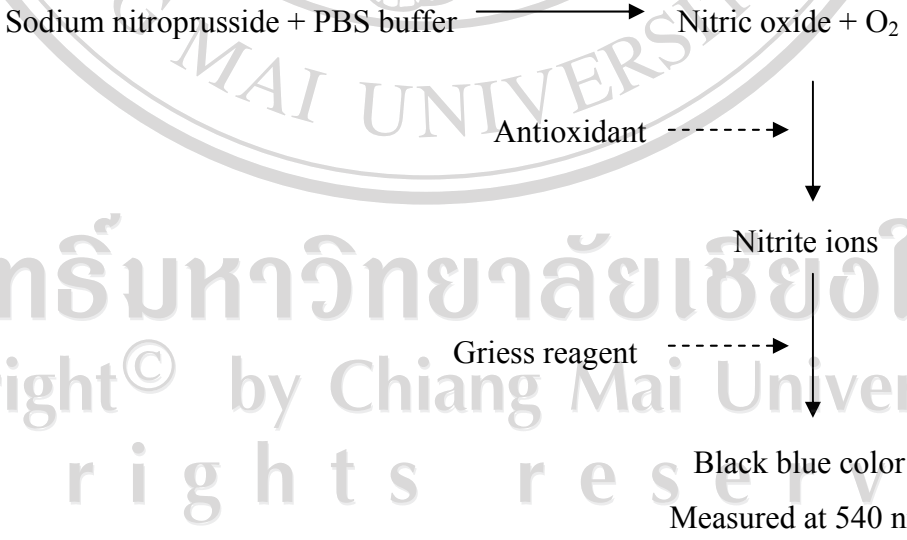
Measured at 560 nm

(Multimode Detector Beckman Coulter model DTX880)

Scheme 13. The protocol of superoxide anion radical scavenging activity assay

3.6.3 Nitric oxide scavenging activity assay

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide. Nitric oxide interacts with oxygen to produce nitrite ions that can be observed by Griess reagent. Scavengers of nitric oxide complete with oxygen leading to reduced production of nitric oxide (Marcocci, 1994). Different concentrations of samples and SNP (5 mM final concentration) in PBS buffer in a final volume of 1 ml were incubated at 25°C for 150 minutes. A control experiment without samples but with equivalent amount of vehicles was conducted in an identical manner of the control. After incubation, 150 µl of the reaction mixtures were removed and diluted with 100 µl of the Griess reagent (1% sulfanilamide and 0.1% naphthyletylenediamine dihydrochloride in 2% H_3PO_4). The absorbance of the chromaphore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was measured at 540 mn. Curcumin was used as a reference standard. Curcumin was a spectrophotometrically detectable compound. The absorbance of chromaphore at the measured wavelength was in a concentration-dependent manner (modified from Sreejayan and Rao, 1997).



The capability of scavenge the nitric oxide radical was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of negative control} - \text{Absorbance of sample}}{\text{Absorbance of negative control}} \times 100$$

The 50% effective concentration (EC_{50}) was calculated according to the relationship of concentration and %inhibition.

Reagents

Sodium nitroprusside (SNP), Standard curcumin, Potassium chloride, Potassium dihydrogen phosphate, Sodium acetate trihydrate, Sodium chloride

Procedure

Sodium nitroprusside was dissolved in PBS pH 7.4

⇓

Nitrite ions + Samples or standard

⇓

Griess reagent

⇓

Measured at 540 nm

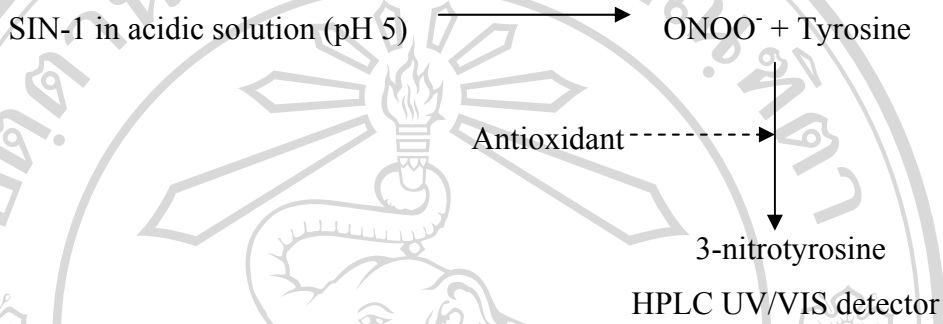
(Multimode Detector Beckman Coulter model DTX880)

Scheme 14. The protocol of nitric oxide scavenging activity assay

3.6.4 Peroxynitrite scavenging activity assay

Peroxynitrite formed with nitric oxide and superoxide anion, is a strong oxidant that damages cell membranes and proteins. SIN-1 is the metabolite from molsidomine. It decomposes spontaneously in neutral aqueous media-consuming oxygen to release NO and the superoxide anion simultaneously. Thus, the reagent can be used as a possible peroxynitrite donor. Acidic stock solution of SIN-1 (1mM) was added dropwise under vigorous vortexing to 1mM tyrosine in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing various concentrations of tested compound (initial 100 μg/ml to 2000 μg/ml),

followed by incubation for 1 hour. Reaction mixtures were kept on ice until HPLC analysis. 20 µl samples were injected onto the C_{18} reversed phase column with 4 mm x 4mm guard column. Elution was with 50 mM KH_2PO_4/H_3PO_4 buffer (pH 3.5) containing 10% methanol at the flow rate 1 ml/min. 3-nitrotyrosine was detected with UV-VIS detector.



Reagents

3-morpholinosydnonimine (SIN-1), 3-nitrotyrosine, L-tyrosine, Phosphoric acid, Potassium dihydrogen phosphate, and Potassium hydrogen phosphate

Procedure

SIN-1 in acidic solution (pH 5) + Tyrosine

⇓

Peroxynitrite + Samples or Standard

⇓

3-nitrotyrosine

⇓

HPLC UV/VIS detector

(Shimadzu class VP)

Scheme 15. The protocol of peroxynitrite scavenging activity assay

3.6.5 Determination of DNA damage protection-induced by Fenton reaction

The reaction mixture contained 50 ng/µl plasmid DNA PUC18 in buffer, 8 µM of Fe(II), 25 µM of H_2O_2 and different concentrations of tested samples. After incubation at 37°C for 60 minutes, 3 µl of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycol) was added to the reaction mixture, then an aliquot (10 µl) was loaded to a 1% agarose gel in 0.5x TBE buffer and electrophoresis was carried out as 100 V for 90 minutes. Following electrophoresis, gels were stained with ethidium bromide for 30 minutes. After washing, the bands were visualized under a UV transilluminator. Changing of the intensity of the bands are due to DNA strand breakage that leads to a decease in the proportion of form I (supercoiled form) and to an increase in form II (relaxed form) and form III (linear form). The relative intensities of DNA bands were determined with a Bio-Rad gel documentation system.

Reagents

Agarose, Ethidium bromide, Plasmid DNA PUC18 , Ferrous sulphate, Hydrogen peroxide, 6x loading buffer, Tris, Boric acid, Ehtylene diamine tetra acetate

Procedure

Plasmid DNA PUC18 + Fe (II) + H_2O_2 + Different concentrations of tested sample

⇓

Incubated at 37°C for 60 minutes

⇓

Loaded to 1% agarose gel

⇓

Electrophoresis was performed 100 V for 90 minutes

⇓

Stained with ethidium bromide

⇓

Visualized under a UV transilluminator

⇓

Determine with gel documentation system

Scheme 16. The protocol of DNA damage protection-induced by Fenton reaction

3.7 Total polyphenol (Folin-Denis method)

This method is used routinely to measure total polyphenol. This experiment is based on the oxidation of phenolic groups with phosphomolybdic and sodium tungstate. After 30 minutes incubation, the oxidation green-blue complex is measured at 725 nm. Gallic acid and pyrogallol were used as standard and the total phenolic content is expressed as gallic acid and pyrogallol equivalents, respectively (AOAC, 1990).

Reagents

Folin-Denis reagent, Sodium carbonate, Standard gallic acid, and standard pyrogallol

Preparation of Folin-Denis reagent

50 g of sodium tungstate, 12 g of phosphomolybdic acid, and 25 ml of phosphoric acid were mixed in 350 ml of water. The mixture was shaken vigorously and boil under a reflux condenser for 2 hours, then cool and diluted with water to 500 ml and keep closed in a refrigerator (2-8^{o}C).

Preparation of sodium carbonate solution

Dissolve 200 g of sodium carbonate anhydrous in 800 ml of water and bring to a boil. After cooling, add a few crystals of sodium carbonate anhydrous and after 24 hours, filter and add water to 1000 ml.

Calibration curve

Dissolve 0.5 g of standard gallic acid or pyrogallol in 10 ml ethanol and dilute with water to 100 ml volumetric flask. To prepare a calibration curve, add 1, 2, 3, 5, 10, and 20 ml of the above standard stock solution into 100 ml volumetric flasks, then dilute

to volume with water. These solutions will have standard concentration of 50, 100, 150, 250, 500, and 1000 mg/ml, respectively.

Procedure

From each calibration solution, sample, or blank, 200 µl in to separate cuvettes, and to each add 1.58 ml of water, then add 100 µl of Folin-Denis reagent and mix well. Add 300 µl of sodium carbonate solution and shake to mix. After 30 minutes incubation, the reaction mixture was determined the absorbance at 725 nm against the blank.

Sample or standard + water

⇓

Folin-Denis reagent was added

⇓

Sodium carbonate solution was added

⇓

Determine the absorbance at 725 nm

(Spectrophotometer Shimadzu model UV-2450)

Scheme 17. The protocol of Folin-Denis assay

3.8 Determination of cyclooxygenase-2 (COX-2) inhibition activity

The sample extracts were evaluated COX-2 inhibition by Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTECH), Pathumthani, Thailand. The procedure tests the ability of compounds to inhibit COX-2 enzyme ability via the measurement of prostaglandin produced from mouse COX-2 null cell line, aspirin was used as the reference standard.

3.9 Statistic analysis.

Where appropriate, Complete randomize design (CRD) or a one way analysis of variance (ANOVA) was used for statistical analysis. Sample means were differentiated using Duncan. Data would show statistical difference when *P* value is below 0.5.