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Original article

Anti-aging and antioxidant of four traditional malaysian plants using simplex centroid mixture design approach

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ABSTRACT

Aging is a naturally biological process with adverse effects. The continuous accumulation of reactive oxygen species (ROS) trigger cellular and tissue damage by activating several aging enzymes. The antioxidant properties of traditional medicinal plants used by Jakun aborigine's community are a promising approach to alleviate aging process and prevent Alzheimer. The aim of the current investigation was to optimize a novel anti-aging formulation from traditional plants (*Cnestis palala* stem, *Urceola micrantha* stem, *Marantodes pumilum* stem and *Microporus xanthopus* fruiting bodies) using simplex centroid mixture design (SCMD). After selecting the optimal formulations based on desirability function of antioxidant activity (DPPH, $ABTS^{+}$ and FRAP), they were further examined against the activity of aging-related-enzymes (collagenase, tyrosinase, acetyl- and butyrylcholinesterase). The single extracts of *C. palala, U. micrantha* and the binary mixture of *C. palala* and *U. micrantha* were the optimal formulations with high antioxidant activities. Single extract of *U. micrantha* showed the highest inhibition towards matrix metalloproteinase-1 (49.44 ± 4.11 %), while *C. palala* water extract showed highest inhibitions towards tyrosinase (14.06 ± 0.31%), acetylcholinesterase (32.92 ± 2.13%) and butyrylcholinesterase (34.89 ± 2.8 4%) enzymes. The single extracts of *C. palala* and *U. micrantha* displayed better activity as compared to the binary mixture formulation. In conclusion, these findings could be a baseline for further exploration of novel anti-aging agents from natural resources.

1. Introduction

Aging is defined as normal biological events that occur throughout human life. Conventionally, aging is accompanied with undesirable events in human organism. Skin conditions which are highly exposed to extrinsic factors (environmental aggressor such as UV radiation and smoking) is in the fourth rank that contribute to the nonfatal disease burden (Tobin, 2017). The changes in appearances such as development of wrinkles, irregular dryness, dark pigmentation, deep furrows, severe atrophy, lesion, laxity are the features of extrinsic aging (Mukherjee et al., 2011). The intrinsic aging or chronological aging is characterized by fine wrinkles formation, irregular hair growth, inability to sweat sufficiently, and reduction of subcutaneous fat tissues especially in the face, and insufficient perspiration (Kammeyer and Luiten, 2015).

Oxidative stress, a biological process increased in brain with aging, occurs due to the imbalance between reactive oxygen species (ROS) and redox system. Alzheimer's disease (AD), a type of neurodegenerative disease, has been associated with the dysfunction of mitochondrial and antioxidant systems (Jayaraj et al., 2020). High concentrations of oxidized proteins and extensive lipids peroxidation have been found in post-mortem brain tissue isolated

Abbreviations: ROS, reactive oxygen species; AD, Alzheimer disease; SCMD, simplex centroid mixture design; TAC, Total Antioxidant Capacity; MMP-1, matrix metalloproteinase-1; AChE, acetylcholinesterase enzyme; BuChE, butyrylcholinesterase enzyme.

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from patients with early stages of AD (Mushtaq et al., 2015). In the absence of pharmaceutical drug, the healthy recommendations are still promoting the consumption of antioxidants to prevent the onset of diseases or reduce the secondary pathologies(Abu Bakar et al., 2016). Based on the cholinergic hypothesis, cholinesterase inhibitors, such as galanthamine, rivastigmine, tacrine and donepenzil, have been considered therapeutic targets (Kumar et al., 2021). There are plenty of traditional plants with high phytochemicals content that exert inhibitory action against ROS and cholinesterase activity (Mukherjee et al., 2007). Few studies showed that there is a correlation between the consumption of supplement rich in antioxidants and aging (Hano and Tungmunnithum, 2020).

Traditional plants, used by aborigine communities, could be a promising agent for developing nutricosmetic products. For example, Jakun community is a group of aborigines that live in Kampung Peta, Endau Rompin National Park, Johor, Malaysia. The traditional phytomedicine of Jakun community has been documented in treating certain diseases such as tuberculosis and malaria (Ismail et al., 2018; Sabran et al., 2016). Also, the females in Jakun community are still consuming several sorts of plants infusion for different healthy purposes. These plants are *Cnestis palala* (Lour.) Merr. (locally known as Pengesep, family: Connaraceae), *Urceola Micrantha* (Wall. ex G.Don) Mabb. (locally known as Serapat, family : Annonaceae), *Marantodes pumilum* (Blume) Kuntze, and a type of fungi, *Microporus xanthopus* (locally known as Kulat kelentit kering, family: Polyporaceae) (Ismail, 2017). The decoction and infusion of these plants and fungi mixture were traditionally consumed by Jakun women for post-partum recovery and boosting energy. The above-mentioned plants have shown high quantity of bioactive compounds with promising antioxidants acitivities (Chua et al., 2012; Dej-adisai et al., 2015; Lar, 2014).

Traditionally, herbal practitioner and skillful healer acquire the knowledge on herbal mixture from their folks. They believe that mixture of herbs could synergize medicinal properties, decrease toxicity and mask the unpleasant taste (Guimarães et al., 2011). An additive effect refers to a combination of different herbs that provides the sum of the effects of the individual components. A synergistic effects happens when the effect is greater than the sum of individual components, whereas an antagonism occurs when the sum of the effect is less than the mathematical sum that would be predicted from individual components (Ndhlala et al., 2010; Wang et al., 2011). Therefore, the current study was conducted to determine the optimal formulations of herbal mixture based on the antioxidant activities using SCMD. The selected optimal formulation was further evaluated for anti-aging and anti-Alzheimer's activities.

2. Materials and methods

2.1. Plants materials

The plant samples were collected from Endau-Rompin Johor National Park in February 2015, with the permit approved by Johor National Park Corporation (JNPC). Voucher specimens (SUNR 100, SUNR 101, SUNR 102 and SUNR 103) were prepared in order to identify the plants and deposited at Repository for UTHM Natural History Gallery.

2.2. Preparation of plants infusions

The fresh roots of plant materials were cleaned and air-dried for two weeks. Then, it was cut into smaller pieces, grind, and sieve to get 500-μm mesh size. The samples were kept in air-tight container at room temperature. For the antioxidant assays, 0.1 g of single or mixture (based on the mixture proportion generated by Design-Expert Software) was infused in 200 mL of hot boiling water (100C). Then, the infusion was stirred for 3 min and left to cool for 10 min at room temperature. Then, the infusion was filtered by using filter paper (Whatman No. 1). For anti-aging assays, 2 g of sample was infused in 200 mL of hot boiling water (100C).

2.3. Design of experiment and mixture optimization

Simplex centroid mixture design (SCMD) was used in current study to achieve the optimum compositions of herbal mixture and to assess the interaction effects between each component. The polyherbal formulations consisted of *Cnestis palala* stem, *Urceola micrantha* stem, *Marantodes pumilum* stem and *Microporus xanthopus* fruiting bodies. In total, 24 formulations (including 5 repetitive formulations) were generated and the responses were analysed using the Design Expert 9 software. The experimental layout of the herbal formulations was displayed in Table 1. The responses (dependent variables) were three antioxidant assays, namely, 2,2-Diphenyl-1-picrylhydrazyl radical assay (DPPH), ferric reducing/antioxidant power assay (FRAP), and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay. The canonical model of Scheffé (1963) was expressed for each response after adjustment being made according to the experimental data and the linear, quadratic and special cubic models were tested to obtain respective regression coefficients (Eq. (1)). Regression coefficients which are not significant were discarded, and data were refitted to obtain the final model for each parameter. Coefficient of determination (R^2) and adjusted R^2 were calculated to determine the adequacy and goodness of fitting for each model.

$$yi = \sum_{(i=1)}^{q} b_i^* x_i + \sum_{(i<j)}^{q} b_{ij}{}^* x_i x_j + \sum_{i<j}^{q} \cdot \sum_{j<k}^{q} \cdot \sum_{k}^{q} b_{ijk}^* x_i x_j x_k + \cdots + b_{12}^* q x 1 x 2 x 9 \quad (1)$$

Where y_i is the response function of the observed data, b_i is the dependent variable with $1 > x_i$ greater than 0 and the Σ =1.0 (Yoshiara et al., 2012). The response surface plot was generated from the adjusted models. The optimization of herbal mixture was determined by using desirability function as proposed by Derringer and Suich (Derringer and Suich, 1980). Each response is set to a certain goal such as maximum, minimum, target, or in range, and the overall desirability calculated ranges from 0 to 1 (least to most desirable) (Kim et al., 2016). The optimal formulations were examined in triplicate in three independent experiments for the purpose of validation. The experimental values were compared with the predicted values estimated by the model within a 95% confidence interval. Three formulations with high desirability functions were selected for anti-aging evaluation.

2.4. Determination of total antioxidant capacity (TAC)

2.4.1. ABTS assay

ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical decolorization assay ABTS decolorization assay was carried out according to method described by (Sabli et al., 2012) with slight modification. The ABTS free radical monocation solution ($ABTS^+$)was generated by reacting ABTS solution (7.0 mM) with potassium persulfate (2.45 mM) to produce blue-green colour of$ABTS^+$. The $ABTS^+$ was stored in a dark room for 16 h to prevent oxidation process. Then, $ABTS^+$solution was diluted with distilled water in order to obtain absorbance of 0.70 ± 0.02 units at 734 nm. Then, 200 μL of plant infusion was added to 2.0 mLABTS +. The mixture was vortexed for 45 s and left for 5 min. The absorbance value was measured at 734 nm by using spectrophotometer. Ascorbic acid was used as positive control in serial concentrations

Table 1
Simplex centroid mixture design and response value.

F*	Factor : Mixture Proportion (mg)				Responses : Antioxidant Activities		
	Cnestis palala	Urceola micrantha	Marantodes pumilum	Microporus xanthopus	ABTS (mg AEAC/L)	DPPH (%)	FRAP (mg /L)
1	100.00	0	0	0	$170.95^{n} \pm 0.71$	$61.59^{j} \pm 2.70$	$251.42^{n} \pm 15.36$
2	0	100.00	0	0	$163.34^{m} \pm 0.17$	$54.27^{g-i} \pm 3.02$	$214.89^{kl} \pm 18.44$
3	0	0	100.00	0	$17.25^{c} \pm 1.94$	$44.27^{d-f} \pm 1.55$	$9.42^{a} \pm 2.33$
4	0	0	0	100.00	$3.82^{bc} \pm 1.05$	$20.65^{ab} \pm 1.63$	$3.40^{a} \pm 1.49$
5	50.00	50.00	0	0	$164.19^{m} \pm 1.21$	$60.56^{j} \pm 4.61$	$238.64^{mn} \pm 12.47$
6	50.00	0	50.00	0	$136.97^{ij} \pm 3.06$	$58.34^{h-j} \pm 0.80$	$141.38^{gh} \pm 5.00$
7	50.00	0	0	50.00	$125.86^{h} \pm 3.48$	$50.74^{f-h} \pm 1.81$	$132.99^{fg} \pm 9.18$
8	0	50.00	50.00	0	$131.79^{i} \pm 4.34$	$52.01^{g-i} \pm 2.23$	$115.96^{e} \pm 3.59$
9	0	50.00	0	50.00	$122.99^{h} \pm 6.67$	$51.97^{f-i} \pm 1.86$	$107.51^{e} \pm 4.90$
10	0	0	50.00	50.00	$13.90^{b} \pm 6.02$	$26.10^{b} \pm 4.68$	$4.95^{a} \pm 2.39$
11	33.33	33.33	33.33	0	$137.16^{ij} \pm 2.70$	$50.99^{f-i} \pm 2.18$	$174.06^{i} \pm 1.53$
12	33.33	33.33	0	33.33	$138.92^{j} \pm 3.06$	$54.63^{g-j} \pm 6.97$	$154.83^{h} \pm 5.16$
13	33.33	0	33.33	33.33	$90.77^{f} \pm 0.64$	$41.22^{cd} \pm 2.11$	$89.48^{d} \pm 4.43$
14	0	33.33	33.33	33.33	$86.42^{f} \pm 2.20$	$51.63^{f-i} \pm 6.68$	$79.6^{cd} \pm 8.61$
15	25.00	25.00	25.00	25.00	$112.99^{g} \pm 6.06$	$51.50^{f-i} \pm 1.94$	$121.48^{ef} \pm 5.64$
16	62.50	12.50	12.50	12.50	$152.44^{l} \pm 4.24$	$54.39^{g-j} \pm 5.14$	$196.38^{j} \pm 4.53$
17	12.50	62.50	12.50	12.50	$146.97^{k} \pm 4.09$	$57.84^{h-j} \pm 5.61$	$176.26^{i} \pm 1.49$
18	12.50	12.50	62.50	12.50	$70.31^{e} \pm 2.10$	$46.97^{e-g} \pm 2.02$	$69.48^{b,c} \pm 5.98$
19	12.50	12.50	12.50	62.50	$61.6^{d} \pm 3.34$	$43.56^{de} \pm 3.31$	$60.79^{b} \pm 3.92$
20	100.00	0	0	0	$165.86^{mn} \pm 1.47$	$56.92^{h-j} \pm 1.94$	$245.80^{n} \pm 5.11$
21	0	100.00	0	0	$163.18^{m} \pm 2.36$	$52.11^{g-j} \pm 2.38$	$209.24^{jk} \pm 10.54$
22	0	0	100.00	0	$19.89^{c} \pm 1.50$	$36.27^{c} \pm 1.90$	$10.27^{a} \pm 2.60$
23	0	0	0	100.00	$2.83^{a} \pm 0.32$	$16.77^{a} \pm 4.96$	$2.69^{a} \pm 0.42$
24	50.00	50.00	0	0	$161.60^{m} \pm 2.39$	$58.78^{ij} \pm 4.03$	$224.89^{lm} \pm 16.91$

Values are expressed as mean ± standard deviation (n = 3). Means with different letters were significantly different at $p < 0.05$ by Duncan's multiple range tests. F*; Formulations.

ranging from 0 to 60 μg/mL. Final result was expressed as mg ascorbic acid equivalent antioxidant capacity per 1 L of sample infusion (AEAC/L).

2.4.2. DPPH (2,2-diphenyl-1-pycryl-hydrazyl) free radical scavenging activity

The antioxidant activities of the infusions were measured in terms of hydrogen donating or radical scavenging ability using the DPPH scavenging activity method as described by Moubayed et al (2017) with slight modifications. About 2.5 mL of sample infusion (0–100 μg/mL) was mixed with 1 mL of DPPH solution (0.3 mM, in methanol) in microplate reader. The reaction mixture was incubated for 30 min at 25C and the absorbance was measured at 517 nm. The percentage of scavenging activity was calculated as in equation (2).

$$Percentage of scavenging activity(\%) = 100 - \left[\left(\frac{A_{sample} - -A_{empty}}{A_{control}}\right) x\ 100\right] \quad (2)$$

where, A_{sample} is the absorbance of test samples (DPPH solution with samples), A_{empty} is the absorbance of samples only (sample without DPPH solution) and $A_{control}$ is the absorbance of control (DPPH solution without sample).

2.4.3. FRAP (Ferric reducing/antioxidant power) assay

The reducing ability of the plants' infusions was assessed based on the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) according to Ouyang et al (2018) with slight modification. Initially, FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6) with 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) and 20 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) with ratio 10:1:1. The FRAP reagent was added to the cuvette and blank reading was taken at 593 nm by using spectrophotometer. A total of 100 μL sample infusion and 300 μL distilled water were added to the FRAP reagent in test tube. After 4 min, a second reading was taken at 593 nm. Ferrous sulphate was run as standard at serial concentrations (0–10 0 μg/mL). A standard curve was prepared by plotting the absorbance value of each standard versus its concentrations. The result was expressed as the concentration of antioxidant having a ferric reducing ability in 1 L of sample infusion (mg/L).

2.5. Anti-aging assays

2.5.1. Collagenase inhibition assay

Collagenase inhibition assay was carried out by using matrix metalloproteinase-1 (MMP-1) colorimetric drug discovery kit (Karim et al., 2014). About 20 μL of sample was diluted with 50 μL buffer-solution (50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35 and 1 mM DTNB in DMSO). A 20 μL of MMP-1 enzyme (*E.coli* recombinant human MMP-1 catalytic domain, 153 mU/ μL) was added to 96-well plate and was incubated at 37C for 30 min. Then, 10 μL of substrate (thiopeptide, Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5; 100 μM) was added to 96-well plate. The absorbance was read at 410 nm and monitored for 10 min. The slope of remaining activity for the tested sample against the control (without sample) was calculated in percentage using formula in equations (3) to (5). The percentage of inhibition was obtained by subtracting the obtained value from 100.

$$\text{Inhibitor activity remaining}(\%) = (V_{inhibitor}/V_{control}) x100 \quad (3)$$

$$\text{Velocity reaction of inhibitor } V_{inhibitor} = \text{Absorbance}_{inhibitor}/\text{min}_{inhibitor} \quad (4)$$

$$\text{Velocity reaction of control } V_{control} = \text{Absorbance}_{control}/\text{min}_{control} \quad (5)$$

2.5.2. Tyrosinase inhibition assay

Overproduction of melanin has been associated with the condition of hyperpigmentation of skin such as melasma and ephelids. Thus, the inhibition of tyrosinase enzyme is reflective of controlling hyperpigmentation and associated conditions. Tyrosinase inhibitory activity was determined spectrophotometrically according

to Neagu et al (2018) with slight modifications. The tyrosinase activity was determined using L-DOPA as a substrate. Briefly, 100 μL of phosphate buffer was dispensed in 96-well plate. A 50 μL of 700 units/mL of mushroom tyrosinase in 0.1 M phosphate buffer (pH 6.8) and 50 μL of sample (with various concentration) were added to the 96-well plate. The plate was pre-incubated at 25 ℃ for 10 min. The reaction was initiated by the addition of 100 μL of L-DOPA (in 0.1 M phosphate buffer pH 6.8) and the incubation was continued for 20 min at room temperature. The amount of dopachrome formation was quantified against blank at 475 nm. Kojic acid was used as standard (positive control). The tyrosinase activity was calculated as in equation (6).

$$\text{Tyrosinase activity}(\%) = [(A\text{-}B)/(C\text{-}D)]x100 \quad (6)$$

Where, A is an absorbance of reaction mixture containing test sample and mushroom tyrosinase; B is an absorbance of blank sample containing test sample but without mushroom tyrosinase; C is an absorbance of reaction mixture without test sample and with mushroom tyrosinase; D is absorbance of the well without both test sample and mushroom tyrosinase (L- DOPA alone)

2.5.3. Acetyl- and butyrylcholinesterase inhibition assay

Acetylcholinesterase (AChE) and butyrylcholinesterase enzymes (BuChE) inhibition assays were done according to Neagu et al (2016) with slight modifications. A 140 μL of phosphate buffers was added to 20 μL samples (with serial concentrations) or standard followed by a 20 μL of acetylcholinesterase enzyme (2 units/mL). The mixture was incubated for 30 min at room temperature. Then, 10 μL of DTNB solution (3.96 mg DTNB, 1.5 mg sodium bicarbonate, 10 mL pH 8.0 phosphate buffer) was added to the mixture. The reaction was initiated by adding 10 μL acetylthiocholine iodide (21.7 mg acetylthiocholine iodide in 10 mL phosphate buffer pH 7.7). The absorbance was read at 412 nm at 25℃. The standard used for acetylcholinesterase inhibition assay was galanthamine. For butyrylcholinesterase inhibition assay, similar procedure was applied by using butyrylcholinesterase as an enzyme, S-butyrylthiocholine iodide as a substrate and eserine as a standard. The percentage of acetyl- and butyrylcholinesterase inhibition was calculated by using equation (7).

$$\text{Percentage Inhibition } (\%) = \left[\frac{(Absorbance_{control} - - Absorbance_{sample})}{Absorbance_{control}}\right] x\ 100\% \quad (7)$$

2.6. Statistical analysis

All experiments were carried out in triplicates for three independent experiments. The data was statistically analysed by using one-way ANOVA and Duncan post-hoc test to test the significant differences between the formulations, with a level of statistical significance set at $p \leq 0.05$ (IBM SPSS Statistic version 20). The result was presented as mean ± standard deviation.

3. Results

3.1. Total antioxidant capacity

The DPPH, ABTS radical scavenging activity and FRAP reducing power of water plant extracts and their combination is presented in Table 1. *Censtis palala* extract (F1) had the highest value of FRAP reducing power (251.42 mg/L), DPPH (61.95 mg/AEAC/L) and ABTS radical scavenging activity (170 mg/AEAC/L). *Microporus xanthopus* extract had the lowest value in ABTS, DPPH and FRAP. The optimal herbal combination in FRAP reducing power, DPPH and ABTS radical scavenging activity was found in *Cinsetis palala* and *Urcelo micrantha* (F5) with 238.64 (mg/L), 60.56 % and, 164 (mg/AEAC/L) respectively. The lowest antioxidant ability exhibited by the binary mixture of *Marantodes pumilum* and *M. xanthopus* (F10).

3.2. Simplex centroid mixture design analysis

The experimental responses data (Table 1) were subjected to analysis of variance (ANOVA) (Table 2) and prediction equation (Table 3) was developed for each response. ANOVA test was performed to analyse the importance of current study and investigat-

Table 2
ANOVA Table of antioxidant activities (ABTS, DPPH and FRAP).

Response variable	Sum of square	Degree of freedom	Mean square	F value	Probability	
ABTS – Special cubic						
Regression	77679.10	11	7061.74	774.14	< 0.0001	Significant
Residual	109.46	12	9.12			
Lack of fit	89.17	7	12.74	3.14	0.1131	Not significant
Pure error	20.29	5	4.06			
Cor total	77788.57	23				
R^2						0.9986
Adj R^2						0.9973
Adeq Precision						77.171
DPPH - Quadratic						
Regression	3138.7	5	627.74	47.28	< 0.0001	Significant
Residual	238.98	18	13.28			
Lack of fit	184.63	13	14.20	1.31	0.4092	Not significant
Pure error	54.35	5	10.87			
Cor total	3377.68	23				
R^2						0.9292
Adj R^2						0.9096
Adeq Precision						23.241
FRAP – Linear						
Regression	162327.55	3	54109.18	1823.67	< 0.0001	Significant
Residual	593.41	20	29.67			
Lack of fit	482.3	15	32.15	1.45	0.3621	Not Significant
Pure error	111.11	5	22.22			
Cor total	162327.55	23				
R^2						0.9964
Adj R^2						0.9958
Adeq Precision						113.875

Table 3
Coefficients of each model fitted, and their level of significance determined by p-value.

Variables	ABTS		DPPH		FRAP	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
A	167.90	< 0.0001**	60.81	< 0.0001**	254.39	< 0.0001**
B	165.52	< 0.0001**	55.11	< 0.0001**	215.28	< 0.0001**
C	18.55	< 0.0001**	42.46	< 0.0001**	14.46	< 0.0001**
D	3.09	< 0.0001 **	18.46	< 0.0001**	3.01	< 0.0001**
AB	–	–	–	–	–	–
AC	174.58	< 0.0001**	–	–	–	–
AD	160.07	< 0.0001**	37.24	0.0153*	–	–
BC	163.56	< 0.0001**	–	–	–	–
BD	158.29	< 0.0001**	72.57	< 0.0001**	–	–
CD	–	–	–	–	–	–
ABC	−429.52	0.0004**	–	–	–	–
ABD	−192.57	0.0530*	–	–	–	–
ACD	−274.47	0.0099*	–	–	–	–
BCD	−314.69	0.0043*	–	–		–

Level of statistical significance : *P < 0.05, **P < 0.0001; A – *Cnestis palala*, B – *Urceola micrantha*, C – *Marantodes pumilum*, D – *Microporus xanthopus*.

ing the validity of regression equation. The independent variable (mixture proportion) and the response variables were fitted to linear, quadratic and special cubic models (Table 2). Quadratic model had been selected to describe the relationship between the factors (mixture proportion) and response (DPPH). Special cubic model was adequate to describe the relationship between the factor and ABTS scavenging activity, while the best model to explain the effect of mixture proportion on FRAP reducing ability is the linear model. Backward elimination had been applied to the quadratic model and special cubic model to remove the insignificant terms in the model. In current study, the R^2 and adjusted R^2 (adj R^2) for all responses were greater than 0.9 which showed the models selected were fit. In mixture design, insignificant lack of fit in the model was favourable as the model need to be fit.

3.3. Effect and interaction of polyherbal formulation on antioxidant activities

The effect of herbal mixture on antioxidant activities were illustrated in the three-dimensional (3D) surface plot generated from the models (Fig. 1). Each point on the plot represents different proportions of components in the mixture. Three components, *Cnestis palala* (A), *Urceola micrantha* (B) and *Marantodes pumilum* (C) have been included in the 3D surface plots, while *Microporus xanthopus* has been set as constant, due to its low antioxidant activity. It was observed that the zone of maximum ABTS [Fig. 1 (i)] was located at the area containing single formulation of *C. palala* (A), *U. micrantha* (B) and the binary combinations of *C. palala* and *U. micrantha* (A-B). Vertex C represented by *M. pumilum* exhibited the lowest ABTS scavenging activity. All the significant binary terms in Table 4 were negative, and this is reflected in the 3D response surface graph in which the curve from the vertex to the midpoint going downward. For DPPH and ABTS, the surface plot [Fig. 1 (ii) & (iii)] showed the flat plane is tipped upward, towards the direction of vertex A which means that the component A contribute more FRAP value as compared to the other component. The simultaneous optimization of herbal mixture using ABTS, DPPH and FRAP results led to three optimal formulations (*C. palala*, binary mixture of *C. palala* and *U. micrantha* and *U. micrantha*) based on the desirability functions of 0.984, 0.879 and 0.878 (Table 4). For the purpose of validation, these formulations were prepared and analyzed using the same experimental assays and the obtained result were compared with the value predicted by the regression model (Table 5). All the experimental data were within the ± 95% prediction intervals. This

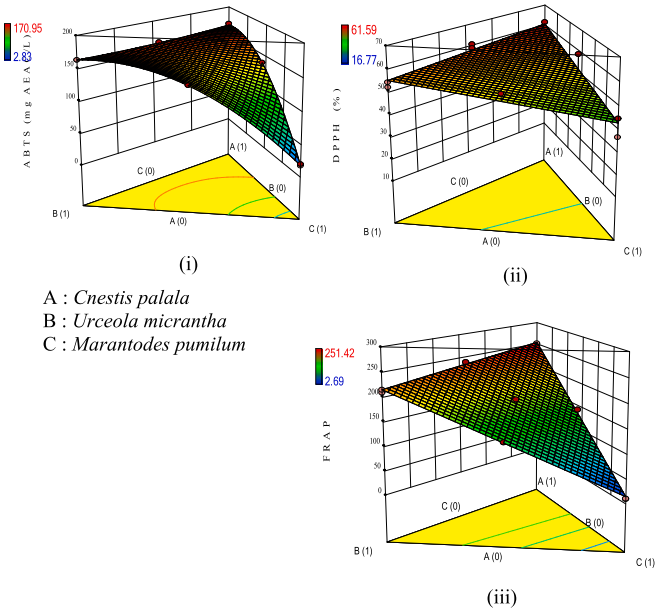

Fig. 1. Surface plot of responses (i) ABTS (ii) DPPH (iii) FRAP.

Table 4
Formulation selection based on desirability function.

Number	Mixture Proportion (%)				Desirability
	C. palala	*U. micrantha*	*M. pumilum*	*M. xanthopus*	
1	100	0	0	0	0.984
2	50	50	0	0	0.879
3	0	100	0	0	0.878

Table 5
Predicted and experimental value of optimal formulations.

Assays	Samples	Experimental value	Predicted value	95% Low prediction interval	95% High prediction interval
ABTS	CP	169.56 ± 1.77	167.90	162.25	173.54
	CPUM	164.20 ± 1.21	165.21	160.58	169.84
	UM	164.23 ± 1.42	162.52	156.88	168.17
DPPH	CP	61.66 ± 2.58	60.81	51.92	69.69
	CPUM	60.56 ± 4.61	57.96	49.79	66.12
	UM	57.12 ± 4.05	55.11	46.22	63.99
FRAP	CP	252.57 ± 15.00	254.39	244.87	263.90
	CPUM	232.10 ± 7.30	234.83	226.76	242.91
	UM	216.38 ± 2.18	215.28	205.76	224.79

Values are expressed as mean ± standard deviation (n = 3). CP refers to *Cnestis palala*, UM refers to *Urceola micrantha*, CPUM refers to binary mixture of *C. palala* and *U. micrantha.*

indicates that the regression models can be regarded as significant and predictive. Thus, the three optimal formulations were further evaluated for the anti-aging activities *in vitro.*

3.4. Anti-aging activities

3.4.1. Anti-collagenase activity

The collagenase inhibition activity of the herbal extracts (*U. micrantha, C. palala*, binary mixture of *C. palala* and *U. micrantha*) was determined at different concentrations (0.25, 0.5, and 1 mg/ml) and elucidated in Fig. 2. Among the three herbal extracts, *U. micrantha* inhibited MMP-1 in a dose–response fashion where the highest value (49.44%) was observed at concentration 1 mg/ml. However, at concentration 0.25 mg/ml, the binary mixture of *C. palala* and *U. micrantha* had similar inhibition activity with *U. micrantha* 7.78% and 10%, respectively. Notably, *C. palala* had higher anti-collagenase activity than the binary mixture of *C. palala* and *U. micrantha* at concentration 1 mg/ml.

3.4.2. Anti-tyrosinase activity

The tyrosinase inhibition capacity of plants extracts, and their combination was compared with standard Kojic acid and the result was present in Fig. 3. At three serial concentrations, the single formulation of *C. palala* and *U. micrantha*, and their binary combination had<20% inhibition of tyrosinase. The single extract of *C. palala* exhibited the highest ability of tyrosinase inhibition followed by the binary combination of *C. palala* and *U. micrantha* in a dose–response fashion. Notably, the single formulation of *U. micrantha* had lower tyrosinase inhibition ability.

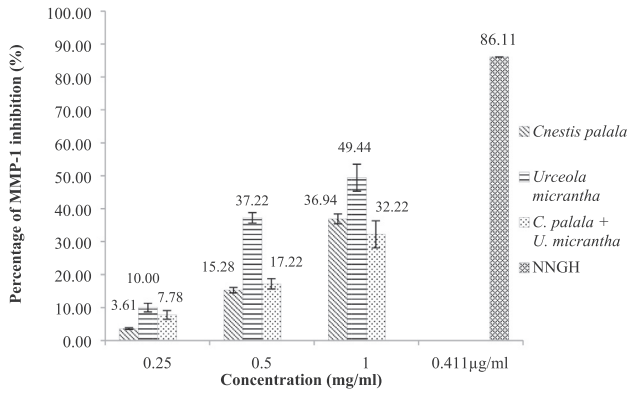

Fig. 2. MMP-1 inhibition of plant extracts compared with NNGH as a s. Values are expressed as mean ± standard deviation (n = 3).

3.4.3. Anti-cholinesterase activity

The inhibitory potential of plant extracts and their binary combination against AChE and BuChE at serial concentration was demonstrated in Figs. 4 and 5. *C. palala* extract showed the highest AChE and BuChE inhibitory as 32.92 ± 2.13 % and 38.88 ± 3.4 % respectively, in the comparison with *U. micrantha* and binary combination of *C. palala* and *U. micrantha* at 2 mg/ml. Notably, the binary combination of *C. palala* and *U. micrantha* showed similar inhibitory activity with *U. micrantha* extract against AChE. At concentration 0.5 mg/ml, the binary combination of *C. palala* and *U. micrantha* inhibited 21.3 ± 2.4% of BuChE while the other plant extracts were between 15 ± 1.5% and 17.31 ± %.

4. Discussion

ROS have been associated with various chronic diseases, including aging, diabetes mellitus, cardiovascular diseases, cancer, and inflammatory arthritis (Ranneh et al., 2017). This association result from the crosstalk between ROS generation and the propagation of cell signaling pathways that lead to the extracellular matrix degeneration and neurodegeneration. Thus, antioxidants have been emerged as preventive measurements for chronic diseases (Ahmed et al., 2017). Initial screening on antioxidant activities of formulations were performed to determine the potential radical scavenging activity by which ameliorating aging effect over skin and brain is possible. Since single type of assay is insufficient to give the conclusive antioxidant ability of the extract (Ranneh et al., 2018), three different antioxidant assays (DPPH˙, ABTS+ and FRAP assays) based on hydrogen atom transfer (HAT) and electron transfer (ET) were used in the current study.

All formulations displayed the ability to scavenge ABTS•+ and DPPH˙ radical and showed reducing ability towards ferric ion in FRAP assay. Among the four medicinal species, only *M. pumilum* and *M. xanthopus* have been assessed for the antioxidant activities

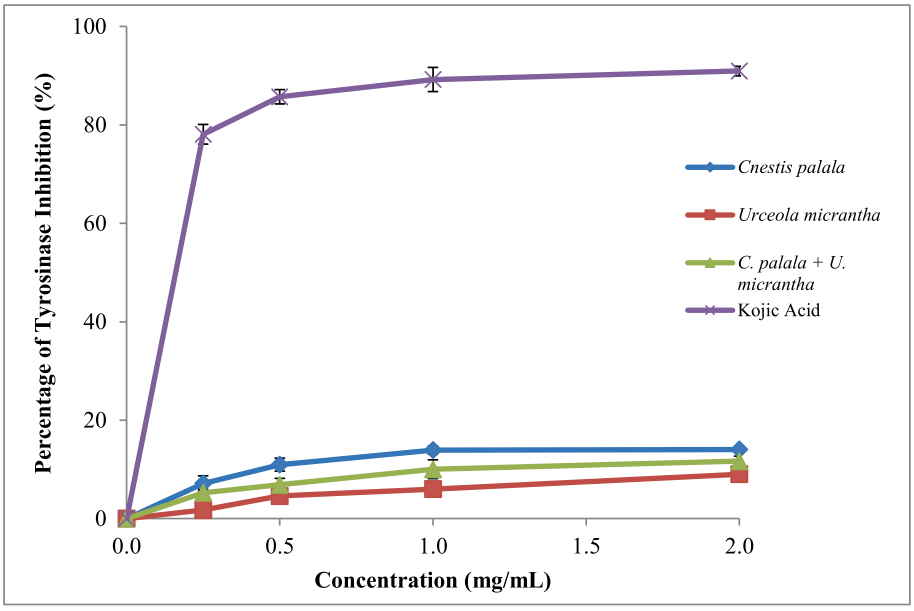

Fig. 3. Tyrosinase inhibition of optimal formulations.

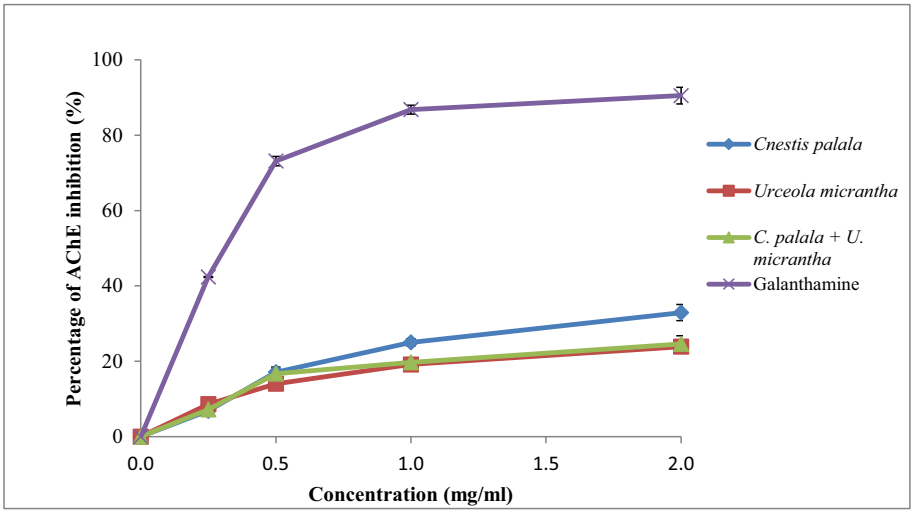

Fig. 4. Acetylcholinesterase inhibition activity of tested formulations. Values are expressed as mean ± standard deviation (n = 3).

in previous study. Tamrakar et al. (2016) demonstrated that the *M. xanthopus* extract displayed 1.2–1.5% inhibition of DPPH radical and 0.14–0.15% inhibition of ABTS, which considered low compared to other species. In contrary, the findings of this study exhibited that single formulation *M. xanthopus* had low antioxidant activities, while *C. palala* had the highest antioxidant potential due to its high content of phenolic and flavonoid (Ismail et al., 2018). The redox properties of phenolic compounds appear as reducing agents, hydrogen donors and singlet oxygen quencher (Atoui et al., 2005). The structure–antioxidant activity relationship for phenolic compounds influenced by the position of hydroxyl group, the presence of other functional groups in the whole molecule, and their conjugation to hydroxyl group (Xu et al., 2016). Thus, the high phenolic content would likely to result in higher antioxidant properties of the plants.

The regression coefficient for all significant terms in the optimized models are summarised in Table 3. Generally, the positive sign in the equation of fitted model indicated the ability of a factor to increase the response variable, while the negative sign showed the ability of a factor to decrease the response (Janser et al., 2013; Ouedrhiri et al., 2016). In the case of ABTS response, the binary mixture of *C. palala/M. pumilum* (AC), *C. palala/M. xanthopus* (AD), *U. micrantha/M. pumilum* (BC), *U. micrantha/M. xanthopus* (BD) displayed synergistic interactions between the medicinal plants as the coefficient was positive. This means that the binary mixture could enhance the ABTS scavenging activity. However, all the ternary mixture contributed negatively to the ABTS scavenging activity which indicated the presence of antagonistic effects when three medicinal plants were combined together.

In the case of DPPH scavenging capacity, binary mixture of *C. palala* and *M. xanthopus* (AD) and binary mixture of *U. micrantha* and *M. xanthopus* (BD) showed significant synergistic effect on DPPH scavenging capacity. Previous study showed that the efficacy of herbal mixture does not result from the potencies of individual herb, but rather form more complex interaction between medicinal plants (Yang et al., 2009). The changes in flavonoid content might make a more significant contribution to the synergism effects of the herbal pair. Yang et al. (Yang et al., 2009) also demonstrated

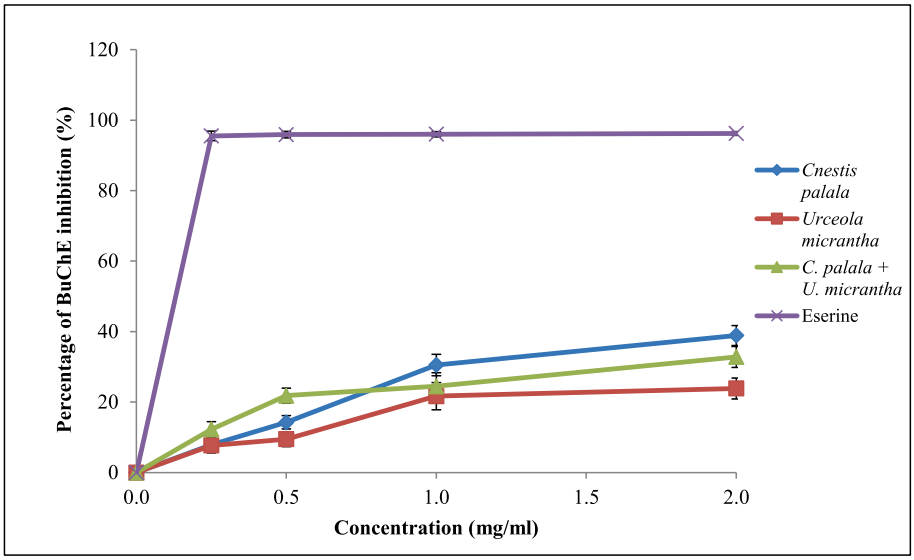

Fig. 5. Butyrylcholinesterase inhibition activity of samples and standard. Values are expressed as mean ± standard deviation (n = 3).

that the eight traditional herb pairs have larger DPPH scavenging capacity compared to its theoretical sum of the respective constituents' herbs (Yang et al., 2009). Current findings suggest that the formulations have different effects towards different antioxidant assays.

The differences in the behavior of the mixture on antioxidant activity could be due to the chemical nature and reactivity of the phytochemical compounds presented in the mixture (Kurin et al., 2012). The individual phenolic compound could undergo structural changes due to polymerization and/or other chemical reactions which ultimately result in variations of antioxidant activities (Pinelo et al., 2004). The radical scavenging capacity of phenolic compound is contributed by the hydrogen-donating ability. The number and position of hydroxyl group in the phenolic compound could affect the scavenging ability of the compound towards free radicals (Yang et al., 2009). In addition, the availability of hydroxyl group depends on chemical structure and spatial conformation, which can alter the reactivity of molecules. However, when the degree of polymerization exceeds a critical value, the increased molecular complexity could reduce the antioxidant capacity due to the steric hindrance, which reduce the availability of the hydroxyl groups (Pinelo et al., 2004; Queirós et al., 2009).

The simultaneous optimization of herbal mixture using the ABTS, DPPH and FRAP led to three optimal formulations based on the desirability functions. These findings suggest that the optimal formulations with high antioxidant activity could be acquired through the single formulation of *C. palala*, followed by binary mixture of *C. palala* and *U. micrantha* and single formulation of *U. micrantha*. Previously, the three formulations displayed a high concentration of phenolic and flavonoid (Ismail, 2017). Zielinski et al. (2015) demonstrated that white tea composition (100%) exhibited the maximum radical scavenging activity and the highest concentrations of epicatechin, epigallocatechin, and epicatechin gallate. Even though Jakun community prepared the medicinal plants in the form of polyherbal mixture, our results indicated that the single medicinal plant for certain species exerted optimal antioxidant activities. Probably, the antagonistic interactions between the medicinal plants affect the bioactive actions. Thus, it was demonstrated that the single formulation of *C. palala* exhibited maximum antioxidant activity as compared to the mixture of four medicinal plants.

Collagen is the most plentiful component in of the extracellular matrix and it form 80 % of the dry weight of the skin. Furthermore, collagens determine the skin physiology through maintaining the structure, retaining the water and support skin integrity (Prabhu et al., 2021). As a person aged, collagen synthesis in skin tissues reduces while collagenase enzyme levels increase (Thring et al., 2009). Collagenase are enzymes categorized as matrix metalloproteinase-1 (MMP-1) and serine protease, respectively which causes the degradation of collagen and elastin within the extracellular matrix, resulting in skin aging and sagging (Thring, 2012). Plants contain various bioactive components that alleviate skin aging effects through various mechanisms such as inhibition of reactive oxygen species, inhibition of UV-induced MMP-1 expression and inhibition of MMP-1 enzyme (Lee et al., 2010). In the current study, the inhibition of medicinal plants against the MMP-1 enzyme was in a dose-dependent manner. The anti-collagenase activity of the studied plant extracts, belonging to *Connaraceae* and *Apocynaceae* families, has not been reported in previous scientific reports yet. The binary mixture of *C. palala* and *U. micrantha* exhibited lower percentage inhibition compared to the single plants extracts which suggest an absence of additive and synergism effects. These effects could result from complex and adduct formation between the bioactive compounds of the medicinal plants. Similarly, the binary mixture of broccoli and soybean was less effective in protecting cardiac cells (Wang et al., 2012). However, Ghimeray (Ghimeray et al., 2015) demonstrated that the mixture of *Punica granatum*, *Ginkgo biloba*, *Ficus carica* and *Morus alba* fruit extracts exhibit higher collagenase inhibition and antioxidant activities compared to the single extract.

Abnormal pigmentation and formation of wrinkles are the major consequence of skin aging. Exposure of skin to UV radiation results in abnormal increase in melanin content, which eventually develop age spot and melasma (Kim et al., 2017). Tyrosinase, also known as polyphenol oxidase, is a copper-containing enzyme by which melanin is formed on the skin (Souza et al., 2012). Currently, kojic acid and arbutin are commercially available products to inhibit tyrosinase. However, the regular usage of these synthetic compounds has been accompanied with several drawbacks including, high cytotoxicity, low stability towards oxygen and water (Kim and Uyama, 2005). Therefore, searching for alternative tyrosinase inhibitor from natural resources with less toxicity is of great interest to scientific communities.

The tyrosinase catalyze the action of o-hydroxylation of monophenols to o-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of diphenolase to o-quinone (diphenolase or catecholase) activity (Alam et al., 2012). In the present

study, the diphenolase activity of the extract was monitored by using L-dihydroxyphenylalanine (L-DOPA) as substrate, and the tyrosinase as the enzyme. However, several studies have shown that certain species in the family of *Apocynaceae* (same family of *U. micrantha*) exhibited tyrosinase inhibitory action. Likewise, the methanolic extract of *Willughbeia coriacea* root (at concentration 500 µg/mL) inhibit 40.7% of tyrosinase enzyme, when L-DOPA was used as substrate, and 34.6% of tyrosinase inhibition when L-tyrosine was used as substrate (Arung et al., 2009). According to Baurin et al. (2002), *Himatanthus sucuuba* (Spruce ex Müll.Arg). Woodson bark and sap extract exhibited 28% and 12% tyrosinase inhibition, while *Parahancornia fasciculata sap* shows 14% tyrosinase inhibition. Both species are from the *Apocynaceae* family. As demonstrated by Neagu et al. (2016), *Pulmonaria officinalis* and *Centaurium umbellatum* aqueous extract exhibited more than 40% inhibition towards tyrosinase enzyme at 1.5 mg/mL concentration. The tyrosinase inhibitory effects of the two plants had been attributed to their flavonoid compositions. In comparison with current data, the *P. officinalis* and *C. umbellatum* displayed higher tyrosinase enzyme compared to *U. micrantha, C. palala* and the binary mixture. Sindhuja et al. (2014) demonstrated that polyherbal mixture containing *Tridax procumbens, Lantana camara, Euphorbia hirta* and *Thevetia peruviana* showed no enhancement in the tyrosinase inhibition compared to the individual treatment of each plant extract. The previous findings were compatible with our results where the mixture of *C. palala* and *U. micrantha* did not improve any enhancement in tyrosinase inhibitory activity compared to the single plants treatment.

Brain tissues of AD patients were characterized by low levels of neurotransmitters including, acetylcholine and butyrylcholine which play important role in autonomic nervous system (Şenol et al., 2010). The administration of AChE and BuChE inhibitors has been emerged as a promising strategy in AD treatment to prevent the hydrolysis of cholinesterase (Prabhu et al., 2021). Both AChE and BuChE are cholinesterase enzyme that functions in metabolizing acetyl, but possess distinct substrate specificity, enzyme kinetic and activity in different brain region (Pisano et al., 2016). Currently, the approved drug by FDA which act as cholinesterase inhibitor are galanthamine, rivastigmine and donepenzil (Mathew et al., 2014). Galanthamine which was used as positive control in the current study, was isolated from the family Amaryllidaceae. The galanthamine bind competitively and reversibly to the catalytic site on AChE which results in the inhibition of acetylcholine breakdown (Ndhlala et al., 2012). However, the usage of synthetic AChE inhibitors has been accompanied with side effects (Ali et al., 2015). Thus, developing natural and safe nutraceutical with suppressing potential on AChE and BuChE has been required. In present study, *C. palala*, binary mixture of *C. palala* and *U. micrantha,* and *U. micrantha* have exhibited inhibitory activity against AChE and BuChE in a dose-dependent manner. To the best of our knowledge, the current study has reported for the first time the cholinesterase inhibitory activity of *C. palala* (family: *Connaraceae*) and *U. micrantha* (family: *Apocynaceae*). However, *Cnestis ferruginea* which is from the same genus of *C. palala*, exhibited AChE inhibition, antioxidant activity and anti-amnesic effects towards scopolamine-induced cognitive impairments in brain of mice (Ishola et al., 2013). Several species in *Apocynaceae* family have shown similar actions on cholinesterase activity such as *Tabernaemontana* spp., *Catharanthus roseus* and *Rauvolfia sumatrana* Jack (Fadaeinasab et al., 2015; Mukherjee et al., 2007; Pereira et al., 2009). In comparison with other species of plants, *Pulmonaria officinalis* and *Centaurium erythraea* Rafn aqueous extracts inhibited acetylcholinesterase 62.12% and 70.21% respectively (Neagu et al., 2016). This indicate that the three formulations of plant extracts used in the current study was relatively lower in the inhibition of AChE and BuChE compared to other species of plants.

5. Conclusion

A simplex-centroid mixture design was developed to evaluate the individual, binary, ternary and quaternary of *Cnestis palala, Urceola micrantha, Marantodes pumilum* and *Microporus xanthopus*. Based on the antioxidant assays followed by desirability function, *C. palala* is the optimal formulation, followed by binary combination of *U. micrantha* and *C. palala* and, *U. micrantha*. Furthermore, *U. micrantha* displayed the highest anti-collagenase activities, while *C. palala* exhibited the highest anti-tyrosinase, acetyl- and butyryl-cholinesterase activity. The high antioxidant capacity displayed by the plant extracts might contribute to the anti-aging potential. Further investigations are needed to model the optimum extraction method and to illustrate the molecular mechanisms of actions.

Availability of Data

The data supporting the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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