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The Anti-Aging Activities against Oxidative Damages of *Rosa roxburghii* and Multi-Fruit Concentrate Drink

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Abstract This research unveils potential of the *Rosa roxburghii* formula drink for anti-oxidation, anti-aging, anti-inflammation, and the reduction of cancer risk. We recruited 40 high risk subjects (i.e., chronic smoker or drinker) and required them to have daily supplement of *R. roxburghii* formula drink over 12 weeks to investigate the long-term effect on health improvement. We discovered that the drink could increase mitochondrial activity (elevated nicotinamide adenine dinucleotide (NADH) level), alleviate inflammatory response by the reduction of tumor necrosis factor alpha (TNF-α) level, and diminish the expression of cancer biomarkers of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and carcinoembryonic antigen (CEA). As a result, this is the first study to elucidate the synergistic effect of *R.roxburghii* and other five kinds of fruits on alleviation oxidative damages.

Keywords: *Rosa roxburghii, anti-aging, anti-oxidation, anti-inflammation, drink*

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1. Introduction

Aging is no doubt a phenomenon that every living organism must experience during lifespan. It can exhibit in many aspects, for example, reduction in tissue regeneration and energy production, and deregulated immune responses on human [1,2,3]. All of these phenomena reduce one's fitness to survive at a later stage of lifespan. Although the mechanism underlying aging is not fully understood, many studies have observed presence of excessive oxidation and inflammation on aging subjects [4]. Therefore, prevention of excessive oxidation build-up and reduction in inflammation status could provide an effective solution to the phenomenon of aging.

Although reactive oxygen species (ROS) play an important role in homeostasis, insufficient removal of ROS leads to many undesired consequences [5]. To date, the presence of many ROS has been identified on human cells including superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^{\bullet-}$) hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) [6]. They are by-products of metabolic reactions taking place in mitochondria [7]. Therefore, mitochondria are usually the primary target of ROS attack resulting in mitochondrial dysfunction or apoptosis [8]. Even though a default anti-oxidation protection is installed on human cells and is activated in response to the presence of ROS, many environmental factors, for instance, smoking, alcohol and food consumption, and air pollution, could trigger production of a great deal of ROS that cannot be efficiently scavenged by the cellular anti-oxidation protection [9,10,11]. Such imbalance of oxidation/anti-oxidation ratio leads to damage to cell membrane, lipid, protein, and DNA [12]. Consequently, extensive apoptosis is induced followed by a series of inflammation responses.

Inflammation is often observed on the subjects suffering from substantial oxidation attack especially aging subjects [13]. In a mouse model, glutathione peroxidase 4 (GPx4) knockout mice exhibited a significant increase in production of ROS upon stimulation as well as expression of pro-inflammatory markers including TNF-α and interleukin 6 (IL-6), suggesting ROS level is closely associated with pro-inflammation [14]. Chronic inflammation, which is a phenomenon of low-grade systemic inflammation, is possibly the result of continuous exposure to ROS [15]. This is because ROS is involved in activation of the NF-κB pathway eventually giving rise to increase in circulating levels of TNF-α, IL-6 and interleukin 8 (IL-8) [13,16]. Such elevated levels of the pro-inflammatory markers are important in the etiology of many aging-

related diseases including cardiovascular diseases, neurodegenerative diseases (e.g., Alzheimer's disease (AD)), and cancers [17,18,19]. Importantly, higher levels of the pro-inflammatory markers are more prominent in elderly [20]. As such, inflammation plays a critical role in aging and affects the health status of elderly.

Nature fruits provide the natural nutrients required for neutralizing oxidation attack and attenuating inflammation [21]. *R. roxburghii* is a native fruit in Southern China and has been used as medicine since 1765 [22]. It is rich in polyphenols, flavonoids, organic acids, triterpenes, amino acids, polysaccharides and essential oils [23,24,25]. All these elements allow *R. roxburghii* to deliver a range of health benefits including anti-oxidation, anti-mutagenesis, anti-inflammation, anti-aging and anti-tumor effects [26,27,28,29]. Notably, *R. roxburghii* has been proved to enhance the expression of anti-oxidative, DNA mismatch repair, and telomere maintenance (i.e., *XRCC5*) genes in macrophage cells, meaning that *R. roxburghii* can improve the anti-oxidative capability, reduce the possibility of cancer formation, and prolong the life span in cells [30]. Noni, *Morinda citrifolia*, is commonly processed into juice and widely used in over 80 countries [31]. Noni juice is rich in vitamin C and total phenols, which are recognized as a promising anti-oxidant [32]. In addition, it has been proved to be clinically beneficial for reducing smoke-induced oxidation of DNA and blood lipid, and scavenging superoxide anion radicals [33,34]. Moreover, sea buckthorn (*Hippophaes rhamnoides*) is also a common ingredient in food, cosmetics, and medicine. It contains several bioactive compounds such as polyphenols, flavonoids, vitamin C, and so on [35]. Regarding the clinical use, sea buckthorn has been used for anti-oxidation, anti-inflammation, chronic disease prevention (e.g., hypertension, cardiovascular disease) [36,37]. Each of these fruits has been reported to be beneficial for improving health status. However, to our knowledge, the synergistic health benefits when they are consumed together have never been investigated on human.

In the present study, we evaluated health benefits of a natural fruit juice concentrate, called RB concentrate, containing extract of *R. roxburghii*, noni, sea buckthorn, pomegranate, apricot, and apple. We performed a clinical study on a cohort of 40 sub-healthy subjects and investigated if their aging-related parameters including oxidation stress, inflammation, and cancer risk indexes can be ameliorated by continuous consumption of the RB concentrate.

2. Materials and Methods

2.1. Participant

Participants in this study were individuals in the age range from 30 to 60 years old. Eligibility criteria included one of the following: regular smoker, consuming over 90 g alcohol per week, at least twice a week sleeping less than 6 hours, exercising fewer than 3 times per week. Exclusion criteria were patients with medication intake for chronic diseases, the levels of plasma glutamate oxaloacetate transaminase (GOT) and glutamic pyruvic transaminase (GPT) were higher than 120 IU/L, a creatinine clearance rate lower than 25 mL/min.

2.2. Sample Preparation

The RB drink here was adopted from beyonde Roxburghii Plus (750 mL), Unilever Thai Trading Ltd., Bangkok, Thailand. The RB contains *R. roxburghii*, apple, pomegranate, apricot, noni, and sea buckthorn concentrates. Note that the 750 mL of RB drink had 542,880 umol/TE of super oxide radical capacity (SORAC) analyzed by the Brunswick's lab [38]. For the placebo, an apple juice supplemented with flavoring substance and citric acid, is similar to RB on both appearance and taste.

2.3. Study Design

A total of 40 participants met the criteria were enrolled in this study. A randomized, double-blind, placebo-controlled trial was conducted. The participants were randomly assigned to placebo group (n = 20) or RB group (n = 20). The participant characteristics were summarized in Table 1. The participants were informed to consume 100 mL of drinks per day after meal for a period of 12 weeks. This study took place in Chia Nan University of Pharmacy and Technology and has received the certificate of approval from Antai Medical Care Cooperation Antai-Tian-Sheng Memorial Hospital Institutional Review Board (IRB) with the TSMH IRB No. 18-147-A, and has been undertaken according to the Helsinki Declaration.

2.4. Serum Biochemical Parameters

The fasting blood of each participant was collected at weeks 0, 4, 8, and 12 for the following analysis of physiological parameters. The blood samples were centrifuged at 2000 ×g for 15 min at 4°C. The clear serum samples were collected and stored at -70°C until tests were performed. The levels of serum biochemical parameters including, TNF-α, NADH and 8-OHdG were monitored every 4 weeks throughout the study. Other parameters in serum, including the levels of GOT, GPT, blood urine nitrogen (BUN), creatinine (CREA) and CEA were detected.

TNF-α (BD OptEIA Human TNF ELISA set, BD Biosciences #555212, CA), NADH (NAD/NADH Quantitation Colorimetric kit, BioVision #K337-100, CA) and 8-OHdG Quantitation (Human 8-OHdG ELISA kit, Mybiosource #MBS267161, USA) were analyzed according to the manufacturers' instructions. GOT, GPT, BUN and CREA were analyzed using automatic analyzer (Hitachi 7180, Japan), and CEA was measured with Roche Cobas e411 (Switzerland).

2.5. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). The comparison of treatments between each specific time point and week 0 were using unpaired and two-tailed Student's t test. The comparisons of the baseline of each parameter were conducted using unpaired and two-tailed Student's t

test between treated groups and placebo at week 0. Differences between treatments were considered as statistical significance when $p < 0.05$ in all cases. The values are reported as mean ± standard error of the mean (SEM).

3. Results

The study was done on 40 subjects. All subjects were randomly arranged into the two testing groups. All the participants completed the 12-week study. The profiles of subjects were listed in Table 1. All subjects had no chronic diseases, such as diabetes, heart disease, hyperlipidemia, hypertension, arteriosclerosis. There were no statistically significant differences in the distribution of age, height, body weight and BMI between placebo and RB groups. The levels of BUN, CREA, GOT and GPT showed no significant differences among the treatment group at week 0 ($p > 0.05$) (Table 2). After 12 weeks consumption, the levels of BUN in two groups were reduced slightly from 0.7 to 1.85 mg/dL without statistical significance. The CREA concentration in blood in two groups maintained a similar level between 0.83 an 0.9 mg/dL throughout the study. A similar trend was found on the detection of GOT and RB did not significantly affect the serum GOT concentration. A reduction of GPT level by 4.7 U/L was found in RB group without statistical significance ($p = 0.18$).

Table 1. Summary of characteristics of trial participants enrolled in the study

		Placebo	RB
Subjects		20	20
Gender (M/F)		10/10	10/10
Age distribution	30-50 [yr]	17	14
	51-65 [yr]	3	6
Age mean (yr)		40.1 ± 11.1	45.2 ± 12.7
Height (cm)		165.1 ± 8.0	162.2 ± 5.4
Weight (kg]		63.2 ± 14.6	65.7 ± 11.6
BMI (kg/m^2)		23.0 ± 4.0	24.9 ± 3.5

The values represent the number of participants in each category, or mean ± SEM ($n = 20$).

Table 2. The changes of serum biochemical parameters of each group during the study

	Placebo		RB	
	Week 0	Week 12	Week 0	Week 12
BUN (mg/dL)	12.1 ± 0.76	11.10 ± 0.69	14.85 ± 0.85	13.00 ± 0.59*
CREA (mg/dL)	0.86 ± 0.04	0.90 ± 0.05	0.89 ± 0.04	0.86 ± 0.04
GOT (U/L)	21.35 ± 1.72	21.30 ± 2.26	20.85 ± 1.23	20.30 ± 1.04
GPT (U/L)	25.65 ± 2.86	25.00 ± 4.92	24.30 ± 2.93	19.60 ± 2.19

All values represent mean ± SEM ($n = 20$).

The NADH levels in placebo group had no significant change (Figure 1). However, consumption of RB gradually increased the NADH level over this study. The NADH level was increased by 6.23 nM at week 4 ($p < 0.001$) and further increased by 7.55 nM at the end of the trial.

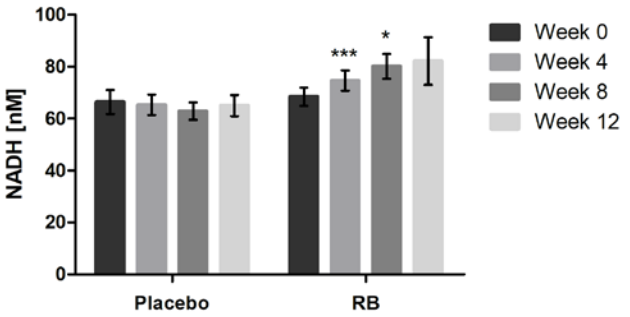

Figure 1. The NADH level in blood. Bars represent mean ± SEM ($n = 20$). *$p < 0.05$, ***$p < 0.001$ vs. week 0 within group.

The levels of serum TNF-α in placebo group over 12 weeks were no statistically significant differences (Figure 2). In the RB group, although the baseline of TNF-α was higher than placebo ($p = 0.02$), an intake of RB for 12 weeks significantly reduced TNF-α level by 1.64 pg/mL over the trial ($p < 0.001$).

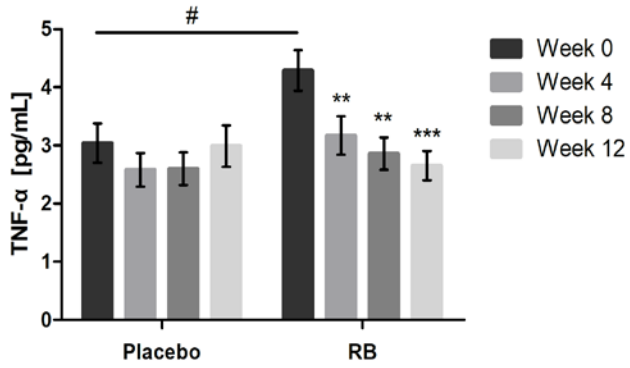

Figure 2. The changes of serum TNF-α over the trial. Bars represent mean ± SEM ($n = 20$). **$p < 0.01$, ***$p < 0.001$ vs. week 0 within group; # $p < 0.05$ vs. placebo at week 0.

The levels of serum 8-OHdG at week 0 for both groups were quite similar (Figure 3). The levels of 8-OHdG in placebo group remained at the almost same levels over the 12-weeks trail. However, RB drinks significantly diminished the levels of 8-OHdG by 1.59 ng/mL ($p < 0.001$).

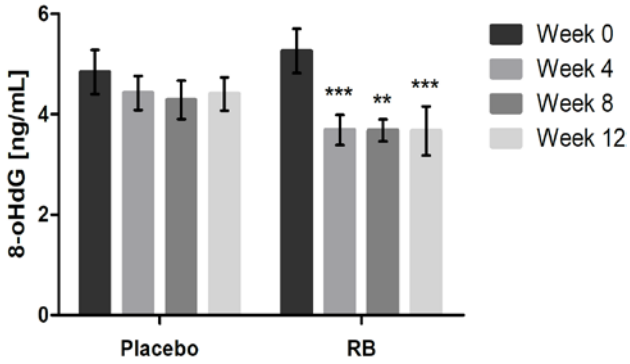

Figure 3. RB consumption reduced serum 8-OHdG level. Bars represent mean ± SEM ($n = 20$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. week 0 within group.

The levels of serum CEA was approximately 2 ng/mL among both groups at week 0 (Figure 4). A 12-weeks consumption of RB resulted in the significant reduction of the levels of CEA by 0.74 ng/mL ($p < 0.001$).

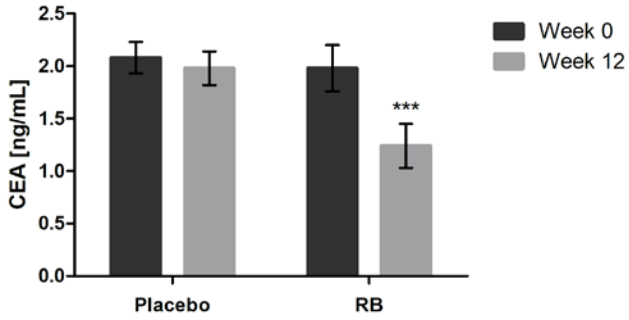

Figure 4. The intake of RB remarkably decreased serum CEA level. Bars represent mean ± SEM (n = 20). ***p < 0.001 vs. week 0 within group.

4. Discussion

In the present study, we attempted to understand the health benefits of the RB concentrate containing the extracts of *R. roxburghii*, noni, sea buckthorn, pomegranate, apricot, and apple. Given deleterious effects of unhealthy lifestyles undermines health fitness expediting aging progress, we performed the clinical study on a cohort of 40 sub-healthy individuals who met the criteria of regular smoker, frequent alcohol consumption, or lack of regular exercise. All these behaviors could contribute to the development of aging-related diseases [9]. The levels of CREA, BUN, GOT, and GPT were monitored over the study and did not show significant difference between before and after the treatment on the individuals who continuously consumed the RB concentrate for 3 months. The results ensure that daily consumption of 100 mL of RB concentrate would not cause adverse effects on kidney and liver.

Mitochondrial activity is highly associated with aging and the RB concentrate can enhance mitochondrial activity. Mitochondria are cellular powerhouse generating energy in the form of ATP utilizing electron flow. NADH plays an essential role in the TCA cycle for ATP synthesis [39]. Unlike other studies which evaluated mitochondrial activity based on NAD^+/NADH ratio, we measured NADH level as an indicator of mitochondrial activity because it is more closely associated with ATP synthesis [40]. The results demonstrated that the continuous consumption of RB concentrate can gradually and significantly elevate NADH level over the course of the study. This suggests that mitochondrial activity and ATP synthesis could be enhanced by the consumption of the RB concentrate. It has been reported that the increase in NADH level due to oral supplement may repair age-associated arterial dysfunction and oxidative damage [41]. Also, free NADH content in mouse young neuron cells is much higher than that of old neuron cells from AD-like mouse brain [42]. Noticeably, in our previous cell study (data not shown; the cell vitality result credited by TCI and TCI Gene groups), we discovered that RB could remarkably increase the mitochondrial membrane activity and mitigate the senescence in human follicle dermal papilla cells. Note that hair growth is a clear embodiment of aging process. Therefore, the consumption of the RB concentrate can possibly improve cellular repair efficiency maintaining cell integrity and functions. Also, the RB concentrate may delay aging and slow telomere attrition and lower risk for developing aging-related diseases, for example, Alzheimer's disease [30].

The RB concentrate can lower pro-inflammation status and possibly delay aging progress. It has been well characterized that elevated pro-inflammation status is often observed on elderly and is associated with diseases [4]. Importantly, suppressing TNF-α level is critical in the reduction of oxidation damage and oxidation-induced aging process. Therefore, we investigated if the RB concentrate can improve one's fitness by lowering the degree of pro-inflammation. The results revealed that continuous consumption of the RB concentrate can significantly decrease pro-inflammation status, as indexed by circulating TNF-α level, by 38% by the end of the trial. Although the results showed baseline discrepancy in circulating TNF-α level between control group and RB group, it is not surprising to observe such difference in a clinical study as the mean circulating TNF-α level can be affected by many factors including age, genetics, and environmental influence. Nevertheless, the decline in the circulating TNF-α level that was observed in only RB group points out a promising health benefits on protecting cells from oxidation attack and delaying the onset of aging characteristics. Additionally, based on the discovery of inverse correlation between TNF-α level and glucose uptake leading to hyperglycemia and hyperinsulinemia in elderly, the RB concentrate could be beneficial for alleviating the symptoms of age-related diabetes or obesity [43]. Overall, the RB concentrate could increase one's fitness by reducing the development of symptoms associated with elevated pro-inflammation status.

The RB concentrate can effectively protect cells from oxidation damage reducing the risk of developing diseases associated with chronic and excessive oxidation. Over-production of ROS by mitochondria can result in damage to cell membrane, lipid, protein, and DNA [44]. In the present study, we demonstrated that the RB concentrate could significantly reduce DNA oxidation damage by 30%. A clinical study has reported the association of DNA oxidation damage and occurrence of cancer, in which 0.26−2.67 nmol/mmol creatinine of 8-OHdG was observed in the individuals without diagnostic breast cancer, whereas the 8-OHdG level on breast cancer patients was in the range of 0.46−6.65 nmol/mmol creatinine [45]. Furthermore, it has been observed that the high levels of 8-OHdG were detected in the urine of the elderly patients with chronic obstructive pulmonary disease or chronic kidney disease high levels of 8-OHdG [46,47]. Therefore, higher 8-OHdG level is one of the characteristics of aging and predisposes ones toward developing cancers. The observation that the RB concentrate can significantly reduce 8-OHdG level indicates that cells can be more effectively protected from oxidation attack. As such, it is expected the continuous consumption of the RB concentrate can lower the risk of developing oxidation-induced diseases to certain degree.

The RB concentrate is beneficial for increasing ones' fitness to survive upon a long period of consumption. The onset of cancers is one of the major causes underlying premature death especially in elderly. Many aging characteristics are highly associated with cancer development. CEA level has been confirmed to be highly

correlated with the cancer stages and oxidative stress level in cancer patients [48,49]. In the present study, we monitored the CEA levels on the subjects who consumed the RB concentrate on a daily basis for 3 months. The positive outcome of significant reduction in CEA level by 37% in RB group and no difference was observed in control group (the CEA levels of all the testing subjects were in the normal reference range of 0-3.8 ng/mL) [50]. These evidences suggest that the RB concentrate can lower the risk of developing cancer to certain extent and improve DNA mismatch repair capacity [30]. Such health benefit provided by the RB concentrate may possibly due to the presence of a broad range of bioactive compounds in the concentrate delivering various cellular protections including, but not limited to, reduction in pro-inflammation status and oxidation damage. This positive outcome warrants a further study in a prospective clinical trial investigating the extent of such health benefits on cancer patients.

5. Conclusion

The synergistic effect of the combination of *R. roxburghii*, apple, pomegranate, apricot, noni, and sea buckthorn concentrates are beneficial for anti-oxidation, anti-inflammation, anti-aging, and cancer prevention. Last but not least, although the exact potent benefits need to be further studied, the anti-oxidative results indicate that RB drink has the potential to prolong the life span of cells by slowing telomere attrition and interfere with the onset of age-associated diseases.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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