Tamarillo Consumption Associated with Increased Acetylcholinesterase Activity and Improved Oxidative Stress Markers in Farmers Exposed to Daily Pesticide-related Activities in Baturiti, Bali, Indonesia

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Abstract

BACKGROUND: The behavior of pesticide use that does not meet the standards will increase the risk of pesticide intoxication among farmers. The main content of pesticides, namely, organophosphates and carbamate, has been widely known to be the main culprit of the negative side effect through inhibition of the acetylcholinesterase (AChE) enzyme. However, this effect theoretically could be reduced by exogenous antioxidants properties provided in tamarillo fruit.

AIM: This study aims to determine the effect of tamarillo consumption on the AChE activity and biomarkers of oxidative stress among farmers who exposed daily pesticide-related activity.

METHODS: A randomized, open-label clinical trial was conducted among 40 farmers in the Baturiti, Tabanan Regency, Bali, Indonesia, during March–August 2018. The respondents were randomly divided into two groups: (1) Groups of farmers without tamarillo juice supplementation (control), and groups of farmers who were given pure tamarillotamarillo juice 250 ml/day every day for 2 weeks (intervention). Measurement of AChE, malondialdehyde (MDA), and superoxide dismutase (SOD) levels was carried out at the beginning and the end of the study. Data were analyzed using SPSS version 17 for windows.

RESULTS: Bioactive compound assessment found several antioxidant properties such as flavonoid, tocopherol, polyphenol, β-carotene, ascorbic acid, citric acid, and anthocyanin. The study participants were dominated by male and the distribution of gender between control and intervention groups was comparable (p > 0.05). There were also no significant differences in age, height, weight, body mass index, land area, duration of being farmers, spraying frequency, and smoking history (p > 0.05). However, bivariate analysis between control and intervention groups found a statistically significant difference in SOD (MD: 23.31 ± 15.89 nmol/l; 95% CI: 14.13–32.49; p < 0.0001), MDA (MD: 81.82 ± 62.45 nmol/l; 95% CI: 45.76–117.88; p < 0.0001), and AChE (MD: 341.61 ± 206.44 nmol/min/ml; p < 0.0001) levels.

CONCLUSION: Tamarillo consumption associated with increased AChE activity and improved oxidative stress through increased SOD and decreased MDA levels among farmers.

Introduction

Indonesia is a developing country which is also one of the largest agricultural countries in the world. Bali, as one among thousands of the small islands, also develops the agrarian sector as one of the main livelihoods of the population in addition to the tourism sector which has grown so rapidly. One type of agriculture in Bali is vegetable farming, which poses a significant risk of pesticides exposure [1]. Activities ranging from preparing and mixing, spraying, cleaning, and treating the leftover pesticides have a high chance of contacting toxic material through the skin/mucosal contact, inhalation, or even ingestion. The risk would even greater with poor adherent to principles of safety and health, which is, unfortunately, the norm for the most farmer on this island [2]. Research by the Hiperkes

and Occupational Safety Center in Bali in collaboration with the Government Agriculture Service in 1998 found that the prevalence of mild poisoning was 20.32%, moderate poisoning 4.25%, and severe poisoning 0.18% [1]. In addition, Sutarga mentioned that the vegetable farmer with mild poisoning was found as high as 23% [3]. Government efforts to prevent/minimized the harmful effects of pesticides have long been carried out, but in reality, the use of pesticides among farmers is still far from safety standards [1], [3], [4].

The main content of pesticides, namely, organophosphates, has been widely known to be the main culprit of the negative effect on health. The main effect of organophosphate is the inhibition of the acetylcholinesterase (AChE) enzyme, which causes increased activity of acetylcholine as a neurotransmitter in synapse or neuromuscular junction [5]. The

effects of toxicity may appear very quickly or up to several hours after entering the body. Severe acute poisoning manifests clinically as increased salivation, vomiting, tremors, and seizures [5], [6]. However, the inhibition of cholinesterase itself cannot explain for the wide range of disorders [7]. Pesticide, primarily organophosphate has been reported to induce oxidative stress through increased production of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), nitrate ($NO3^-$), and nitrite ($NO2^-$) [8], [9]. The presence of oxidative stress is characterized by increased plasma levels of malondialdehyde (MDA) and reduced erythrocyte superoxide dismutase (SOD) enzymes [10], [11], [12], [13].

Oxidative stress can be prevented or mitigated by various antioxidants [14], [15], [16]. Antioxidants can come from food or supplements (exogenous antioxidants) and also produced within the human body itself (endogenous antioxidants). Sources of antioxidants derived from food, generally derived from fruits. One of the fruits that are rich in antioxidants is tamarillo.[17] Tamarillo or Dutch Eggplant (*Cyphomandra betacea*) is a commodity that was cultivated by several regions in Bali. At relatively low prices, this fruit is rich in antioxidants, including Vitamin C, Vitamin E, beta-carotene, anthocyanin, flavonol, phenolic acids, and other flavonoids [17]. These natural exogenous antioxidants could theoretically counter the effects of various free radicals [18].

The authors expect that the consumption of tamarillo fruit in the form of juice will be able to reduce oxidative stress in farmers who exposed to various activity related to the use of pesticide as the previous study [19]. Therefore, this study aimed to evaluate the effect of tamarillo consumption on the AChE activity and oxidative stress markers among farmers exposed to daily pesticide-related activity in Baturiti, Bali, Indonesia.

Methods

An experimental study with the randomized, open-label clinical trial design was conducted in the Baturiti, Tabanan Regency, Bali, Indonesia, during March–August 2018 period. This study involved 40 participants who randomly divided into two groups, groups of farmers without tamarillo juice supplementation (control), and groups of farmers who were given tamarillo juice every day for 2 weeks (intervention). Participants from both groups conduct their daily routine as usual, including pesticide related-activities without an appropriate personal protective device during the study period. The socio-demographic data of the participants were documented before the study began. All of the study protocol had been approved by the joint ethics committee of clinical study Universitas Udayana/Sanglah General Hospital, Denpasar, Bali, Indonesia (Letter no. 2017.03.1.0928).

Tamarillo juice was prepared from fresh fruits. The fruit was cleaned and washed to remove any residual compost using tap water. These were then cut into pieces and put on the blender with added water to produce a total volume of 250 ml. No additional sugar was added to the mixture. A preliminary study has been carried out to assess the bioactive components provided from tamarillo fruit from Biochemistry and Nutrition Laboratory, Faculty of Agricultural Technology, Universitas Udayana with number 07/Lab BN/VIII/2017.

The measurement of MDA, AChE, and SOD levels was carried out at the before and after the study period. The blood samples were taken from peripheral veins, stored in tubes at $0^{o}C$ and immediately sent to the laboratory. The blood samples were analyzed in the Biochemical Laboratory of Universitas Gajah Mada, Yogyakarta, Indonesia.

Serum activity of AChE was assayed using high-performance liquid chromatography. MDA was measured by thiobarbituric acid reactive substances reaction in acidic medium, which on heating formed a pink complex that absorbed maximally at 532 nm. The serum SOD activity was determined by the colorimetric method based on the ability of SOD to inhibit the auto-oxidation of epinephrine at a pH of 10.2. MDA and SOD were assessed in nmol/l, whereas AChE in nmol/min/ml.

Demographic data and laboratory result were statistically analyzed. Continuous data were expressed as mean ± standard deviation or median (minimum-maximum) according to its distribution. The mean difference between the groups was analyzed using T-test or its non-parametric equivalent if it does not fulfill the requirement. Normality test conducted with Shapiro–Wilk and the homogeneity of variance was analyzed with the Levene test. Categorical data were expressed as a percentage and analyzed with Chi-square or Fisher's exact test. All statistical analysis conducted with SPSS for Windows version 17. The threshold of a significant difference is considered at $p < 0.05$.

Results

The preliminary study on major bioactive substance from a crude extract of single tamarillo fruit is shown in Table 1.

A total of 32 participants were committed to the study protocol until the completion of the study protocol. Eight participants were dropped out of the study, one from the control groups and seven from the intervention groups. The study participants were

mostly male (84.37%) in both control (78.94%) and intervention (92.31%) groups (Table 2). The mean ages and the anthropometric measurement, including weight, height, and body mass index, were comparable for both groups (p > 0.05). Most of the participants in the intervention group was graduated from elementary school (46.15%) or did not attend any formal education (42.11%) in the control group. The participants

Table 1: Bioactive compound assessment on a single tamarillo fruit

Parameters	Methods	Unit
Flavonoid	Spectrophotometry	4.33 mg/100 QE bb
Tocopherol	Spectrophotometry	4.61 mg/100 g bb
Polyphenol	Spectrophotometry	71.92 mg/100 g (GAE) bb
β-carotene	Spectrophotometry	628.625 mg/100 g bb
Ascorbic acid (Vitamin C)	Titrimetric	31.02 mg/100 g bb
Citric acid	Titrimetric	2.55% bb
Anthocyanin	Spectrophotometry	0.98 mg/100 g bb
Water level	Gravimetry	83.79% bb
Ash level	Gravimetry	1.01% bb
Lipid	Soxhletation	2.94% bb
Protein	Titrimetric	1.11% bb
Carbohydrate	By difference	11.15% bb
Calorie	Calculation	75.52 kcal

of the study had become a farmer for two decades, with the majority of its land were owned by themselves in both control (52.63%) and intervention (61.54%) groups. The area of lands occupied was varied, with mostly owned, some rented, or in combination. On average, the control (2500 [400–27,500] m^2) and intervention (2750 [100–25,000] m^2) groups showed a comparable median of land area (p = 0.948) (Table 2). The median frequency

of spraying pesticide was 2 times a week, with a median duration of an hour each, which also comparable for both groups (p > 0.05) (Table 2). Furthermore, no differences in the distribution of smoking habit during spraying were observed (p = 0.961). The baseline characteristic of the study participant is summarized in Table 2.

The result of laboratory measurement of each biomarker at pre-study and post-study is shown in Table 3. Our study found that the post-study SOD level was significantly increase in the intervention group (57.96 ± 12.84 nmol/l) compared with control group (38.23 ± 7.87 nmol/l) (p < 0.0001). There was a significant reduce of post-study MDA level in the intervention group (27.27 ± 3.42 nmol/l) compared with control group (110.73 ± 9.68 nmol/l) (p = 0.003) (Table 3). In addition, a significant increase of post-study AChE level was found in the intervention group (375.15 ± 209.99 nmol/min/ml) compared with control group (30.06 ± 4.74 nmol/min/ml) (p < 0.0001) (Table 3).

Discussion

Toxic effect of pesticide on both animals and humans has long been established [18], [19], [20].

Table 2: Baseline characteristics of study respondents

Variables	Control group (n=19)		Intervention group (n=13)		p-value
	Mean ± SD or median (min-max)	n (%)	Mean ± SD or median (min-max)	n (%)	
Gender (%)					
Male		15 (78.94)		12 (92.31)	0.625[a]
Female		4 (21.06)		1 (7.69)	
Age (years)	47.16 ± 11.16		45.38 ± 10.06		0.650[b]
Height (m)	1.64 ± 0.06		1.63 ± 0.06		0.739[b]
Weight (kg)	60.58 ± 10.82		61.34 ± 7.72		0.830[b]
Body mass index	22.29 ± 3.42		22.84 ± 2.72		0.631[b]
Level of education (%)					
Elementary School		6 (31.58)		6 (46.15)	
Junior High School		4 (21.05)		1 (7.69)	
Senior High School		1 (5.26)		3 (23.08)	
Bachelor Degree		0 (0.00)		0 (0.00)	
Not Graduated		8 (42.11)		3 (23.08)	
Land ownership					
Owned		10 (52.63)		8 (61.54)	
Rent		4 (21.05)		2 (15.38)	
Combination		5 (26.32)		3 (23.08)	
Land area (m^2)	2500 (400–27,500)		2750 (100–25,000)		0.948[c]
Duration being farmer (years)	20.53 ± 10.91		21.92 ± 9.44		0.710[b]
Spray frequency (times/week)	2 (1–4)		2 (2–3)		0.448[c]
Duration of spraying (hs)	1 (0.25–2)		1 (0.30–3)		0.596[c]
Smoking (%)					
Yes		6 (31.57)		4 (30.77)	0.961[a]
No		13 (68.43)		9 (69.23)	

[a]Fisher exact test; [b]Independent sample t-test; [c]Mann–Whitney U-Test. Statistically significant if p<0.05.

Table 3: The SOD, MDA, and AChE levels comparison between pre- and post-study

Parameter	Classification	Between groups difference			p-value[d]
		Control (mean ± SD)	Intervention (mean ± SD)	95% Clc (Lower-Upper)	
SOD	Pre-study	38.70 ± 15.77	34.54 ± 11.94	(14.13–32.49)	<0.0001
	Post-study	38.23 ± 7.87	57.96 ± 12.84		
	Mean Difference[a]	−0.45 ± 17.57	23.31 ± 15.89		
	p-value[b]	0.887	<0.0001		
MDA	Pre-study	112.18 ± 43.63	109.09 ± 63.34	(45.76–117.88)	0.003
	Post-study	110.73 ± 9.68	27.27 ± 3.42		
	Mean difference[a]	−1.45 ± 41.60	−81.82 ± 62.45		
	p-value[b]	0.917	<0.0001		
AChE	Pre-study	31.93 ± 5.64	33.54 ± 4.41	(222.41–460.80)	<0.0001
	Post-study	30.06 ± 4.74	375.15 ± 209.99		
	Mean difference[a]	−1.87 ± 5.68	341.61 ± 206.44		
	p-value[b]	0.193	<0.0001		

AChE: Acetylcholinesterase (nmol/min/ml); SOD: Superoxide dismutase (nmol/l); MDA: Malondialdehyde (nmol/l). [a]Mean of difference between post-study value and pre-study value from each subject. [b]p-value derived from paired T-test between pre and post value on each group. [c]CI: Confidence interval of the mean difference between pre-test and post-test assessment in the intervention group. [d]p-value derived from independent T-test of the mean differences between control and intervention groups.

The behavior of pesticide use that does not meet the standards will increase the risk of pesticide intoxication among farmers. The WHO estimates 250,000 deaths occur among 3 million cases of pesticide poisoning every year [21]. Organophosphate pesticide intoxication has been mainly attributed to inhibition of cholinesterase activity. Most farmers have experienced symptoms of poisoning after spraying, such as headaches, nausea, eye irritation, and tremors [3]. However, the inhibition of cholinesterase itself cannot explain for the wide range of disorders, especially in the case of chronic exposure in subclinical dose [7], [18]. Based on those mentioned above, several biomarkers have been elucidated to determine the effect of organophosphate on health, risk of malignancy, and metabolic diseases [22], [23], [24]. So that, the recent studies start to shift focus on other long-term effects of the pesticide such as the oxidative stress as well as the cholinesterase activity among farmers who are more vulnerable to the daily-pesticide exposure. Pesticide, primarily organophosphate containing compound, has been reported increase the production of ROS including H_2O_2, $NO3^-$, and $NO2^-$, which collectively caused a state of increased oxidative stress [8]. When exposure to pesticide persists for a long time, the antioxidant sources can be depleted [25].

Oxidative stress depleted our antioxidant activity, primarily decrease in some enzyme which acts as an endogenous antioxidant. Damage induced by oxidative stress primarily occurs through the production of ROS, which involves "stealing" electrons from nucleic acids, lipids, and proteins, leading to the altered function and structure within the cells [26], [27]. Antioxidants can protect cells from damage caused by ROS and RNS [15]. If the body is exposed to large amounts of ROS, then the level of an endogenous antioxidant such as SOD will decrease [28]. SOD is an enzyme that plays a role in repairing cells and reducing cell damage caused by superoxide radicals. The activity of SOD in individuals with high oxidant exposure is significantly lower compared with that in healthy persons, which probably fuel the development of oxidative stress-related health problems [25]. On the other hand, oxidative stress allows lipid peroxidation in cell membranes with the resulting MDA can be measured in plasma [11], [12]. In fact, MDA is one of the most widely used biomarkers and is suitable for lipid peroxidation among reactive aldehydes [13]. The previous study has confirmed that patients with pesticide intoxication also showed an increased MDA level [10]. Thus, the presence of oxidative stress is characterized by reduced erythrocyte superoxide dismutase (SOD) enzymes and increased plasma levels of MDA.

This study shows that supplementation of tamarillo juice showed significantly higher SOD and AChE, but lower MDA as compared to the control group. This result supports the previous study of Surajudeen *et al*. and Vidyasagar *et al*. Both studies also reported low AChE and SOD activity on the pesticide-exposed worker [9], [19]. Numerous studies have shown that AChE was reduced in farmer exposed to pesticide [29], [30], [31], [32], [33], [34], [35], [36], [37], [38]. Our study also showed a similar result, which further emphasizes that lower AChE is a common feature in pesticide-exposed individuals. AChE has been used for years as the biomarker of neurotoxicity from environmental and occupational exposure of organophosphorus and related compounds. It is a sensitive indicator of chronic exposure due to low recovery rate, therefore, shows cumulative inhibitory effect, and it mirrors the biological effect of the central nervous systems variant of AChE. AChE thus far showed as useful biomarkers as it easy to measure, show a dose-dependent characteristic to exposure level, and exhibits a link to clinically significant adverse effect [39], [40]. However, there is still no single range of value or consensus criteria for defining chronic exposure case.

The previous study also found that pesticide intoxication showed a negative correlation between the levels of cholinesterase and MDA. Meanwhile, positive correlations were found between cholinesterase and catalase and glutathione, which the latter two were endogenous antioxidants [41]. In this study, the antioxidants enzyme activities of SOD were lower in the control group. Decrease of the antioxidant enzyme may be related to rapid consumption and exhaustion of storage of this enzyme in combating free radicals generated due to pesticide exposure. Besides, activities of other major antioxidant enzymes may also be inadequate. A similar condition was also observed in condition with increased exposure to free radical [9]. The observed higher level of MDA in the intervention group compared with control in this study might suggest increased lipid peroxidation in the latter. This indicates that the higher MDA level will lead to the higher the risk of tissue damage due to oxidative stress.

Overall, our result of 2 weeks of tamarillo juice supplementation showed a positive increment of antioxidants enzyme. The biological mechanisms are not entirely clear but may be attributed to the singular or synergistic action of several bioactive compounds. Tamarillo contains several types of antioxidants, including Vitamin C, Vitamin E, beta-carotene, anthocyanin, flavonol, phenolic acids, and other flavonoids [42]. These natural exogenous antioxidants could theoretically counter the effects of various free radicals, such as hydroxyl radicals, superoxide anions, peroxy lipid radicals, nitric oxide, singlet oxygen, and peroxynitrite [17]. A large amount of evidence shows that bioactive compounds such as carotenoids, flavonoids, phytoestrogens, and dietary fiber, which are present in a wide variety of fruits and vegetables, might reduce oxidative stress and inflamation [43], [44], [45], [46], [47], [48], [49]. Tamarillo has been demonstrated to a contained variety

of phenolic composition [50]. Polyphenols are capable of neutralizing reactive species, due to their favorable number and position of hydroxyls. Dietary nutrients rich in polyphenols (e.g. flavonoid) could play an important role in the prevention of biochemical alteration due to oxidative stress, perhaps through its role in increasing the circulation of antioxidant compounds [25].

Tamarillo fruits are considerably nutritious because of its high content of vitamin, phytochemicals including beta-carotene anthocyanins, flavonols, phenolic acids, and large amounts of ascorbic acid [50], [51]. This fruit is rich in antioxidants at relatively low prices; however, the public does not widely acknowledge the positive effect on the various system, both related and unrelated to the antioxidant activity. To date, tamarillo remains unexplored, to the best of our knowledge; this is the first study to evaluate the protective effects of tamarillo on farmer who highly exposed to environmental pollutant like pesticide. Throughout the study period, there were no toxic signs such as vomiting, diarrhea, or constipation detected in the intervention group. Therefore, it could be considered safe. We acknowledge that this study has several limitations. This study was not designed to detect difference level of the effect associated with any type of pesticide nor its specific substance that known to inhibit AChE such as organophosphates or carbamates directly. Therefore, we cannot provide the answer to whether tamarillo could decrease the negative effect of organophosphate better than organocarbamate or vice versa. In addition, this was a preliminary study, thus further study with a larger participant and a more extended period of intervention needed to confirm the result.

Conclusion

Tamarillo consumption showed its potential to protect the body from oxidative stress. In this study, tamarillo associated with increased SOD and the decrements of oxidative stress biomarkers MDA as well as normalization of serum AChE. Therefore, consumption of tamarillo on a daily basis can be recommended as prevention of oxidative stress-related to chronic pesticide exposure.

Acknowledgments

Authors' Contributions

IMM and KT are responsible for the conceptual framework, data gathering, interpreting the results, draft and approving the final manuscript. IPYP and KAW are responsible for statistical analysis and English writing of the manuscript.

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