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Synergistic Antioxidant Effect of *Cinnamomum verum* and Stingless Bee Honey

Arul Mozhi Padmanabhan [1], Doss VA [1,*]

[1] Department of Biochemistry, PSG College of Arts and Science, Coimbatore, India
* Correspondence: victordoss64@gmail.com;victorarokiadoss@psgcas.ac.in(Doss VA);

Scopus Author ID 6505916759

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Abstract: Free radicals are formed based on oxidative damage, and antioxidants block these detrimental molecules from cellular damage. To scrutinize the synergistic antioxidant efficacy of CV and SBH with Total Flavonoid Content (TFC), respectively TFC and *in vitro* antioxidant properties were determined in the aqueous extracts of CV, SBH, and its integration. TAC and FRAP were tested to ascertain the antioxidant status. Both the enzymatic antioxidants such as CAT, SOD, GPx, GR, and non-enzymatic antioxidants such as Vitamins (A, C, and E) and reduced glutathione were investigated. Aqueous CV+SBH extract found to have high TFC, TAC and FRAP (33.01±0.742mg QE/g,3.17±0.410mg AAE/g and 11.01±0.366 mg AAE/g) than in CV (23.80±0.548 mg QE/g, 2.58±0.963mg AAE/g and 10.3±0.792 mg AAE/g) and SBH (15.39±0.846mg QE/g,1.44±0.360mg AAE/g and 9.05±0.405mg AAE/g) extracts. Additionally, enzymatic and non-enzymatic activities of CV+SBH were better than that of the CV and SBH. Finally, aqueous extracts of CV+SBH had a significantly better correlation between TFC and antioxidant activity than the discrete extracts of CV and SBH. From the above findings, we suggest that the synergistic competence of CV+SBH against oxidative stress would inevitably impede the process of free radical/pro-oxidant-induced pathologies.

Keywords: oxidative stress; free radicals; flavonoids; enzymatic antioxidants; vitamins.

1. Introduction

Free radicals are atoms with unpaired electrons and are less stable. Free radicals are formed based on oxidative damage, and antioxidants obstruct these detrimental molecules from cellular damage. ROS serves a dual purpose by being both constructive and destructive. ROS can positively impact cellular responses and immune functions at minimal concentrations; however, a higher degree of ROS can trigger oxidative stress, jeopardizing cellular integrity.

Antioxidants can abolish free radicals by suppressing the synthesis of their intermediary molecules. Oxidative stress is caused by a lack of antioxidants or the inhibition of antioxidant enzymes, damaging or killing cells. When there is an adverse equilibrium between free radical production and antioxidant defenses, this situation is termed oxidative stress. Phenolic compounds are exemplary antioxidants with redox properties that include free radical absorption, neutralization, quenching, and disintegration [1].

Cinnamon is a tropical tree whose inner bark is solely used to spice foods. *Cinnamomum verum*, also known as Ceylon or true cinnamon, is a member of the Lauraceae family. Chronic dietary consumption of *Cinnamomum cassia* (Chinese cinnamon) triggers hepatotoxicity and carcinogenicity exposed to high rates of coumarin content, in contrast to

Cinnamomum verum (CV) [2].CV has been shown to have a multitude of health-promoting and pharmacological properties [3] such as anti-microbial activity [4], anti-larvicidal [5] and anti-inflammatory properties [6], lowering the risk of cardiovascular disease [7], ailing dental problems [8], strengthening cognitive function in Parkinson's disease [9], COVID-19 [10] and slowing the progression of cancer [11].

Stingless Bee Honey (SBH) from Melipona and Trigona contains a lot of water and is slightly more acidic than classic honey [12]. According to findings, SBH is efficacious in a wide range of maladies [13], such as bacterial infections [14], inflammation [15], fertility treatment [16], diabetes [17], and anxiety [18].

As per Rapola *et al*., consolidating the complex mixture of phytochemicals in the targeted sample extracts provides a better defensive impact on human health than the solitary phytoconstituents [19]. The purpose of this research is to support the claim that the combination of CV barks and Stingless bee honey-do indeed have a synergistic effect. Using various *in vitro* models, we were able to ascertain the enzymatic and non-enzymatic antioxidant activities of *Cinnamomum verum* barks, Stingless Bee Honey, and their amalgamation.

2. Materials and Methods

2.1. Sample collection, authentication and preparation of the extract.

The whole plant of *Cinnamomum verum* was collected and authenticated (BSI/SRC/5/23/2019/Tech/465) at the Botanical Survey of India, Southern Regional Centre, Coimbatore. In December, dried barks of *C.verum* were purchased from a local market in Coimbatore, Tamil Nadu. This was ground coarsely and stored in an airtight container.

Stingless bee honey (SBH) was collected from the organic market of Kodai hills, Tamilnadu. This was stored in an airtight container at room temperature at 25±2°C.

2.2. Extraction.

10 grams of dried barks of *C.verum* and Stingless Bee honey were weighed separately. To each added 100 ml of Distilled water was taken separately and plugged with cotton. After 72 hours of room temperature and continuous stirring, the sample was extracted by filtration [20].

Extracted samples of CV bark powder and SBH were infused in a 1:1 ratio and vortexed in the same tube. This extract is used to assess the synergistic potential of the combination.

2.3. Total Flavonoid Content (TFC).

Pipetted out 0.5-1 ml of samples and standard solution to a series of test tubes. Quercetin (100 µg/ml) was used as standard. Added 0.3 ml of 5 % Sodium Nitrite and incubated for 10 minutes, followed by the addition of 0.3ml 10% Aluminium chloride and allowed to stand for 6 minutes. Finally, added 2 ml of 1N NaOH and yellow-colored developed was read at 510nm after incubating for 5 minutes. The total flavonoid content was represented in mg of Quercetin equivalent/gram (QE/g) of sample extract [21].

2.4. Total Antioxidant Capacity (TAC).

The prepared concentration of standard & sample extracts in the range of 50-250µg. Ascorbic acid served as the positive control. 3ml of Phosphomolybdenum reagent was added

and incubated at 97ºC for 90 minutes. After cooling, the absorbance was measured at 695nm. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE) [22].

2.5. Determination of Ferric Reducing Antioxidant Power (FRAP).

A series of Ascorbic acid as standard and aqueous extracts of samples in varying concentrations of 40-200 µg were taken. Added 2.5ml of Potassium ferricyanide and allowed to stand for 30minutes at 50ºC; added 2.5ml of TCA and centrifuged for 10minutes. Collected 2.5 ml of supernatant and added water of equal volume. Now added 0.5ml of Ferric chloride solution and Perl's Prussian blue colored complex was read at 700nm [23].

2.6. Enzymatic antioxidant activity.

Enzymatic antioxidant activities of catalase [24], Superoxide Dismutase [25], Glutathione Peroxidase [26], and Glutathione Reductase [27] were determined in the aqueous bark extracts of CV, SBH, and CV+SBH.

2.7. Non-enzymatic antioxidant activity.

Estimations of non-enzymatic antioxidants such as Vitamin A [28], Vitamin C [29], and Vitamin E [30] were carried out in the aqueous extracts of CV barks, SBH and CV+SBH.

2.8. Statistical analysis.

GraphPad Prism Version 8 was used for statistical processing and representing of data. Data were processed with independent triplicate experiments and represented as mean± Standard Deviation (SD). The correlation of TFC and Antioxidant activity were analyzed using linear regression.

3. Results and Discussion

3.1. Total Flavonoid Content (TFC).

Secondary metabolites such as phenols, flavonoids, tannins, etc., have been used to heal a wide range of health issues. Figure 1 shows the total flavonoid content of the aqueous extracts of *C.verum* bark, SBH, CV+SBH extract, and Quercetin. The total flavonoid content, represented in mg of Quercetin equivalent/gram (QE/g) of sample extract, was found to be maximum in CV+SBH extract, with 33.01±0.742mg of QE/g of extract, succeeded by CV extracts with 23.80±0.548 mg of GAE/g of extract, SBH extract, with 15.39±0.846mg of QE/g of extract and least in Quercetin, with 10.744±0.383mg of QE/g of extract. Utilization of dietary foods enriched with flavonoids, cardiovascular disease has an inverse relationship with mortality rate[31]. Flavonoids are the phenolic compounds which suppress lipid peroxidation and lipoxygenases.

3.2. Total Antioxidant Capacity (TAC).

TAC is assessed to check the degree of inhibition or termination of the oxidative reactions of biomolecules. Maximum TAC at 250 µg was shown by CV+SBH (3.17±0.410mg AAE/g) than the standard Ascorbic acid (2.78±0.981mg AAE/g) followed by CV

(2.58±0.963mg AAE/g) and SBH (1.44±0.360mg AAE/g) in the aqueous extracts were represented in figure 2(A).

At acidic pH, the transition of Mo(VI) in the extract tends to result in the subsequent formation of green phosphate-Mo(V). Figure 2(A) illustrates the maximum potency of antioxidants constituting the different bioactive constituents in CV and SBH was demonstrated by prospective synergism countering free radical-induced oxidative stress.

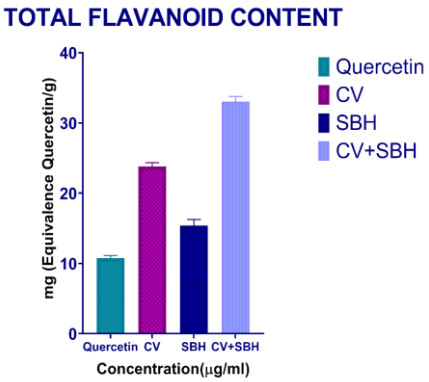

Figure 1. Estimation of Total Flavonoid Content (TFC) of aqueous extracts of CV, SBH, and CV+SBH against Quercetin

3.3. Determination of Ferric Reducing Antioxidant Power (FRAP).

The FRAP method is used to determine the reducing ability of the antioxidants present in the aqueous extract of CV, SBH, and CV+SBH. The reducing power of the sample extract was shown higher in CV+SBH (11.01±0.366 mg AAE/g) than the standard Ascorbic acid (10.22±0.121 mg AAE/g), followed by CV (10.3±0.792 mg AAE/g) and SBH (9.05±0.405mg AAE/g) in the aqueous extracts were represented in figure 2(B).

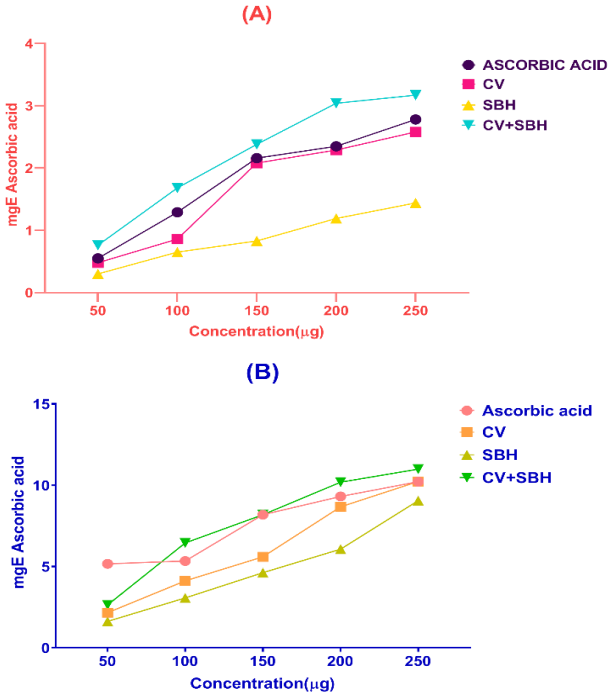

Figure 2. Antioxidant activity of (A) Total Antioxidant Capacity (TAC) and (B) Ferric Reducing Antioxidant Power (FRAP) in the aqueous extract of CV, SBH, and CV+SBH

The reducing power of the aqueous CV+SBH extracts increased substantially than CV and SBH extracts. The reduction potential from ferric (Fe^{3+}) to ferrous (Fe^{2+}) form is marked by the intensity of color formation. This precisely indicates the existence of hydrophilic polyphenols, which have a strong reducing power capacity [32]. The concentration-dependent Ferric reducing antioxidant power pattern observed is shown in Figure 2(B).

3.4. Determination of enzymatic antioxidants.

The antioxidant activity, both enzymatic and non-enzymatic, may shed light on the coping strategy of such free radicals. The enzymatic antioxidant activities evaluated in the aqueous extract of CV, SBH, and CV+SBH were represented in Figure 3. Catalase (CAT) activity was determined to be 83.5±0.377U/mg of protein for CV+SBH, 71.43±0.221U/mg of protein for CV, and 51.4±0.136U/mg of protein for SBH (Figure 3A). Superoxide dismutase (SOD) enzyme activity was found to be the utmost by CV at 9.88±0.489 mmol of H2O2 consumed/min/mg protein, respectively, as shown in Figure 3(B).

SOD, a metalloenzyme and catalyzes the decomposition reaction of superoxide radical ions into water and oxygen. Blockade of antioxidant enzymes results in reduced antioxidant levels, which is a strong indicator of oxidative stress [33]. H_2O_2 is a toxic compound that leads to non-hazardous bioactivity of the cells. The enzyme catalase acts spontaneously and breaks down H_2O_2 oxygen and water molecules. The main locations for the formation of H_2O_2 as a result of ß-oxidation and oxidative stress are considered to be peroxisomes [31].

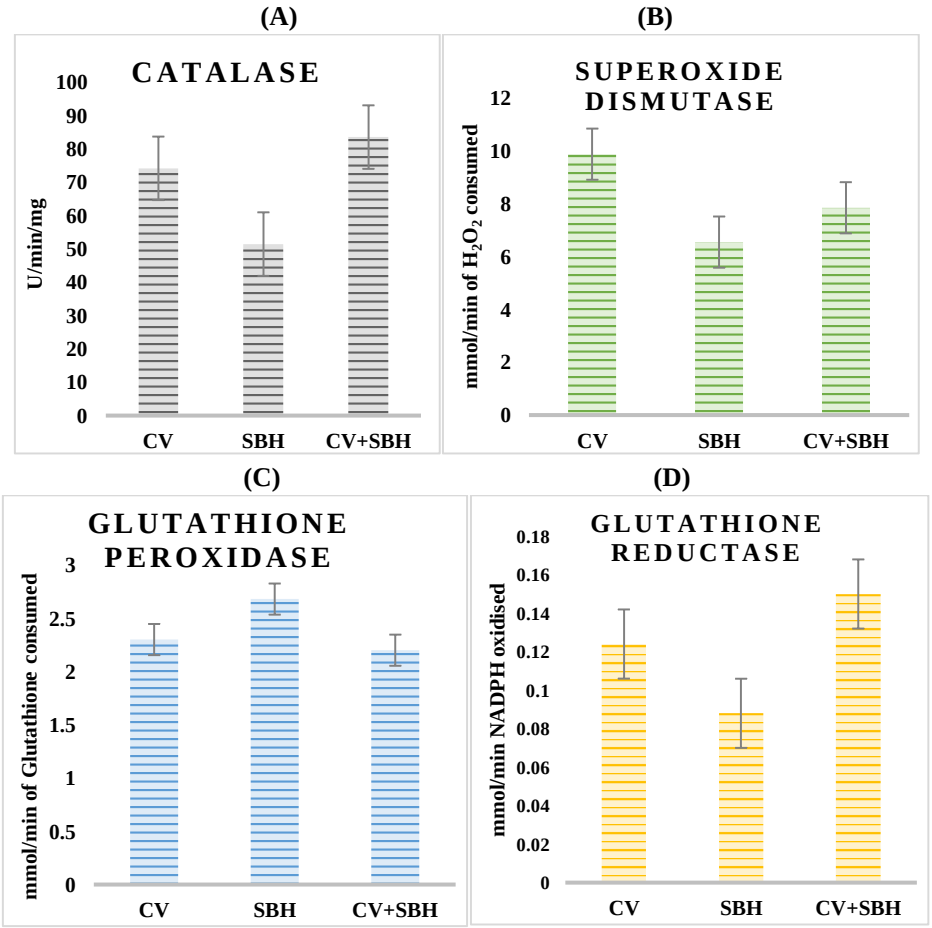

Figure 3. Enzymatic antioxidant activities (A) Catalase, (B) Superoxide Dismutase, (C) Glutathione Peroxidase, and (D) Glutathione Reductase in the aqueous extract of CV, SBH, and CV+SBH

The Glutathione Peroxidase (GPx) activities of aqueous CV barks, SBH, and CV+SBH were found to be 2.3±0.428, 2.68±0.375, and 2.01±0.650 mmol of glutathione consumed/min/ml, respectively (Figure 3C). Finally, Glutathione Reductase (GR) activity was expressed as 0.124±0.741 for CV, 0.088±0.879 for SBH, and 0.15±0.633 for CV+SBH of mmol of NADPH oxidized/min/ml (Figure 3D).

Under stress environments, the GPX heme-containing enzyme is a powerful quencher of reactive intermediate forms of O_2 and peroxy radicals [34]. During mild oxidative stress, GPx acts as the primary defender and competes with catalase for H_2O_2 [35]. Retainment of typical GSH/GSSG ratio levels in cells, disulfide formation in GSSG is preferentially catalyzed by GR, a flavin-containing enzyme [33]. Decreased levels of Glutathione Peroxidase are one of the diagnostic indicators of Oxidative stress.

3.5. Determination of non-enzymatic antioxidants.

The quantities of vitamins present in *C.verum*, SBH, and their combinatorial extract are shown in Figure 4. Vitamin A is an ideal source of retinol, which is determined to be about 0.233±0.144mg/100g in CV,0.148±0.151mg/g in SBH, and 1.482±0.198mg/g in CV+SBH. Vitamin C, with an average of 4.697±0.151mg/g in the extract of CV+SBH, 3.442±0.260mg/g in CV, and 2.588±0.216mg/g in SBH is a water-soluble antioxidant that plays an important role in signaling mechanisms and collagen synthesis. Vitamin E is available at higher concentrations of 3.737±0.178mg/g for CV+SBH, 2.782±0.123mg/g for CV, and 0.760±0.174mg/g for SBH is a lipid-soluble antioxidant and a strong peroxyl radical scavenger [32]. Intake of these dietary vitamins is required to restore these important antioxidants.

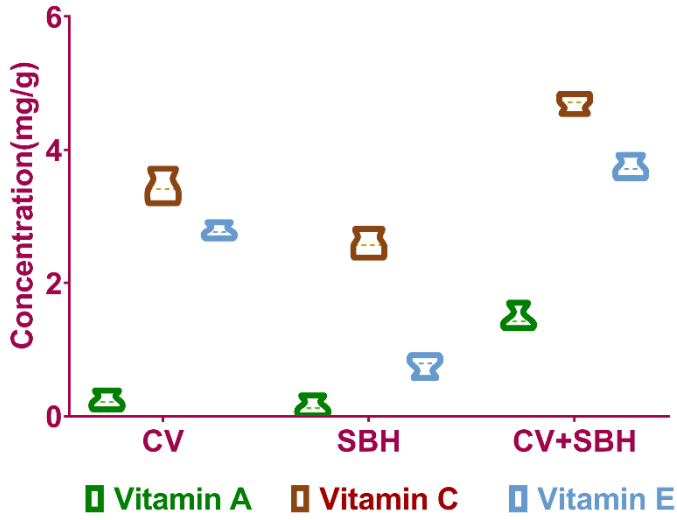

Figure 4. Quantification of Non-enzymatic Antioxidants. Estimation of Vitamin A, C, and E in the aqueous extracts of CV, SBH, and CV+SBH

3.6. Correlation relationship with antioxidant and flavonoid content.

Several studies have found a linear association between total phenolic and flavonoid content and antioxidant capacity [36]. Flavonoid compounds deplete free radicals through donating hydrogen atoms, and thereby, these compounds are considered to be effective antioxidants. The correlation of total flavonoid content with antioxidant capacity and reducing power is shown in Figure 5.

A strong correlative relationship between antioxidant activity and flavonoid content (TAC, R^2 = 0.9994; FRAP, R^2 = 0.9969) was observed at a 95% confidence level. It is fair to

assert after the flavonoid content plays a significant role in the antioxidative mechanism by equating the correlation coefficients (R-Values) that the CV+SBH extract.

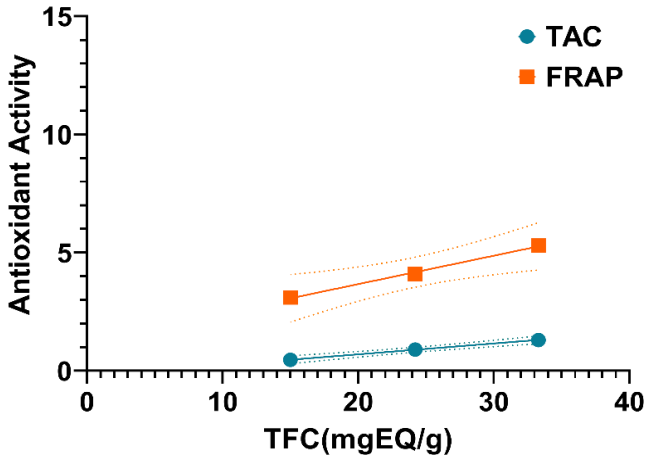

Figure 5. Correlation analysis of TFC vs. Antioxidant activity in aqueous extract of CV, SBH, and CV+SBH by linear regression

4. Conclusions

From this study, aqueous extracts of *Cinnamomum verum* barks, Stingless Bee Honey, and their concoction were reliably pre-eminent in their antioxidative characteristics than the separate extracts. A strong correlation between flavonoid and antioxidant potential of the sample extract was evidenced. Combinatorial bioactive constituents could augment the curative competence for a spectrum of ailments. The potential agonistic nature of these phytonutrients can be extrapolated for the cardioprotective properties exhibited by the combined extract of *Cinnamomum verum* barks and Stingless Bee Honey to get rid of the oxidative stress-mediated cardiac diseases.

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Conflicts of Interest

The authors declare no conflict of interest.

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