

## **ANTIOXIDANT ACTIVITY AND IMMUNOMODULATORY EFFECT OF BLACK TEA INCORPORATED WITH RED GRAPE POMACE EXTRACT SWEETENED BY STEVIOSIDE**

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### **ABSTRACT**

Red grape pomace extract (RGPE) was chromatographically analyzed for its individual phenolic and flavonoid compounds. This extract was also characterized regarding its total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities in terms of scavenging DPPH free radicals. The TPC of RGPE was 31.37 mg of gallic acid equivalents (GAE)/g of extract, and TFC was 3.76 mg of quercetin equivalents/g of extract. Some phenolic compounds including ellagic acid, pyrogallol, catechin, benzoic and cinnamic were identified. Also, identification of TFC appeared high amount of rutin and quercitrin. Tea infusions from black tea incorporated with RGPE and sweetened with stevioside were investigated. The antioxidant activities DPPH scavenging capacity and total phenolics were determined in water extracts of formulated tea infusions, stevioside and black tea. Also, in this study, Phagocytosis inhibition test was carried out to measure immunomodulatory activity of RGP extract, stevioside, black tea and formulated tea infusion. Immunological tests showed that the treatments significantly increased phagocytosis of macrophages. Depending on sensory evaluation, the formulated tea infusion showed overall acceptance compared to normal tea. So, it could be conclude the suitability of red grape pomace extract and stevioside are suited as a supplementation in the production of health promoting beverage.

**Keywords:** Grape pomace, stevioside, tea, antioxidant, phagocytosis, sensory.

### **INTRODUCTION**

Grapes are one of the most widely grown fruit crops throughout the world, and their composition and properties have been extensively investigated. The skins and seeds of grapes are known to be rich sources of phenolic compounds, both flavonoids and non-flavonoids, Poudel *et al.* (2008). They are a good and cheap sources of high quality of polyphenolic compounds which can be used in different therapeutic procedures with the purpose of free radical neutralization in biological systems, Lafka *et al.* (2007) and Makris *et al.* (2007). An important effect of flavonoids is the scavenging of oxygen-derived free radicals. In vitro experiment systems showed that flavonoids posses anti-inflammatory, antiallergic, antiviral, anticarcinogenic, and antimutagenic activities, Teissedre *et al.* (1996), Middleton (1998), Robert *et al.* (2001) and González-Paramás *et al.* (2004). Red grape pomace has also been characterized as a promising source of polyphenolics, most of the phenolic compounds in grape by-products are flavonoids and the grape pomace extract can be used as food additives, natural food colorants, or

health supplements, Kammerer *et al.* (2004) and Liang *et al.* (2008). Gaynor (2012) concluded that ingestion of grape pomace extracts at levels as high as 17.8 mg/kg bw/day is safe.

Tea is one of the most widely consumed beverages worldwide, second only to water. It contains a variety of active phytochemicals with biological properties that promote human health and help reduce the risk of chronic diseases such as allergies, insomnia, headaches, anxiety, intestinal disorders, depression, and high blood pressure, therefore, the tea is popular functional beverage in the world and has gained more and more attention for its health beneficial properties. Among common teas, black tea is consumed more than green tea and oolong tea worldwide, since black tea consumption accounts for 78% of the overall tea beverage industry, Craig (1999), Pan *et al.* (2013) and Li *et al.* (2013)

Stevioside, an abundant component of *Stevia rebaudiana* leaf, has become well-known for its intense sweetness (250–300 times sweeter than sucrose) and is used as a non-caloric sweetener in several countries. Based on direct observations on human and animals, it has been proven that stevioside had to be non-mutagenic, non-teratogenic and non-carcinogenic effects. Stevia has been consumed by human beings for centuries without any negative effects and had approved for use as a sweetener by the Joint Food and Agriculture Organization / World Health Organization Expert Committee on Food Additives. Apart from the sweet taste, *S. rebaudiana* with its secondary plant constituents also offers therapeutic benefits, having antihyperglycaemic, anti-hypertensive, anti-inflammatory, antitumour, anti-diarrhoea, diuretic, and immunomodulatory actions, Chatsudthipong & Muanprasat (2009), Anton *et al.* (2010) and Roberto *et al.* (2012).

In this context, the aim of the present work was to design a functional beverage combining the health-promoting properties of black tea with the phytochemicals of red grape pomace extract and stevioside as a natural sweetener. The antioxidant activity, immunomodulatory effect and the sensory evaluation of tea formulated were also studied.

## **MATERIALS AND METHODS**

### **Materials:**

Red grape fruits (*Vitis vinifera* L, C.V. Crimson) were obtained, during 2012 season, from the local market at Zagazig City. The fruits were washed with water, handpicked from the clusters. The berries were rinsed with distilled water, dried with filter paper, crushed and squeezed to get their byproduct. The prepared grape pomace concentrate, GPC, was dried in a vacuum oven at 40°C and ground into fine powder using laboratory electric mill (Braun, model 2001, DL Germany), then stored in plastic bags in the freezer at -20°C until the extraction was carried out. Stevioside crystalline, white powder, with sweetness power of 300 times as sucrose, procured from Stevia International Company for Agro-industry Product, in Cairo, Egypt (SICAP) was used. Commercial Lepton black teabags were purchased from local supermarket. Ethanol, Sodium carbonate and methanol were obtained

from El-Gomhoreya Co., Cairo, Egypt. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and Folin-Ciocalteus phenol reagent were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA).

**Preparation of red grape pomace extracts:**

The dried red grape pomace (RGP) was extracted with conventional solvent extraction procedure. Ten grams were extracted with 100 ml of alcoholic solvent (ethanol/water 80/20, v/v) in closed vessels by stirring at room temperature, 25°C, for 4 hrs, according to Peschel *et al.* (2006) and Katalinic *et al.* (2010), followed by filtration through Whatman No. 1 filter paper and the residual tissue was washed with 2 x 25 ml of solvent. All vessels were wrapped with aluminium foil to prevent light degradation during extraction. The filtrates were combined in a total extract, which was dried by vacuum rotary evaporator at 50°C (Büchi-water-bath-B480, Switzerland), and freeze dried (Thermo Electron Corporation-Heto Power Dry LL 300 Freeze Dryer, Czechoslovak). The dried extracts were weighed and redissolved in 50% methanol reaching a volume of 25 ml and centrifuged at 5000 rpm for 10 min. The obtained extracts were used for spectrophotometric and HPLC measurements.

**Method of analysis:**

**Determination of total phenolic content (TPC)**

TPC was measured by a UV spectrophotometer (Jenway UV-VIS Spectrophotometer, UK), based on a colorimetric oxidation/reduction reaction as described by Skerget *et al.* (2005) and Folin-Ciocalteu reagent AOCS (1990). Specifically, 0.5 ml of diluted extract (10 mg in 10 ml solvent) was mixed with 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2 ml of Na<sub>2</sub>CO<sub>3</sub> (75 g/l) were added. The final solution was incubated at 50°C for 5 min, then cooled and afterwards the absorbance of the solution was measured at 760 nm. A similar procedure was followed using a distilled water reagent blank and allowed to stand for 2 hrs. Quantification of TPC was based on a gallic acid standard curve generated by preparing 0, 5, 10, 15, 20, 30 mg/l of gallic acid, and total phenolics were expressed as mg gallic acid equivalent (GAE) per gram of red grape pomace extract.

**Determination of total flavonoid content (TFC)**

TFC of the grape pomace extract was determined by the method of Zhishen *et al.* (1999). Briefly, 1 ml of 25-fold diluted extract was added into a 10 ml volumetric flask containing 4 ml of water. At zero time, 0.3 ml of 5% NaNO<sub>2</sub> was added to the flask. After 5 min, 0.3 ml of 10% AlCl<sub>3</sub> was added into the flask. At 6 min, 2 ml of M NaOH was added to the mixture. Immediately, the reaction flask was diluted to volume by the addition of 2.4 ml of distilled water and thoroughly mixed. Absorbance of the pink coloured mixture was read at 510 nm versus the prepared water blank. Six different concentrations of quercetin solution (20-100 mg/l) were used for calibrations. The final results were expressed as mg quercetin equivalent (QE) per gram of RGP extract.

**Identification of phenolic acids and flavonoids compounds by HPLC**

The phenolic acids and flavonoids of the dried extracts were identified according the method described by Goupy *et al.* (1999) and Mattila *et al.* (2000), respectively, using HPLC instrument (Hewlett Packard series 1050, USA) equipped with auto sampling, injector, solvent degasser, UV detector set at 330 nm and quarter HP pump (series 1050). Column C18 hypersil BDS with particle size 5  $\mu\text{m}$  was used. The separation was carried out with methanol and acetonitrile as a mobile phase at flow rate of 1 ml/min. The column temperature was performed at room temperature, throughout the experiment.

Identification and quantification were carried out based on calibrations of the standards prepared from phenolic acid and flavonoid acid from Sigma Co., and dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used for calculation of phenolic and flavonoid compounds by the data analysis of Hewlett Packard Software.

**Preparation of the infusions:**

The infusions were prepared using mixture of dried material. Each drink was made with 100 mL of simmering water (one cup). The mixture includes: 2 gm black tea fortified with 0.02 % of red grape pomace extracts according to recommended level for beverage mentioned by Gaynor (2012) and sweetened with stevioside according to the relation ratio to sucrose.

**Phagocytosis inhibition assay:**

As mentioned before, the red grape pomace extracts, black tea and stevioside were used to formulated infusion tea, the immunomodulatory effects of this material and their mixture (formulated tea) were studied, *in vitro*. The technique adapted by Soliman and Attia (2007) was followed. Briefly, preparation of 0.1% nitroblue tetrazolium (NBT) solution (5mg NBT was dissolved in 2.5 ml 0.9% NaCl protected from light by wrapping the tube) was used. Then the tube was placed in a conical plastic centrifuge tube, shaken until dissolved (30 to 60 unites). Then, 2.5 ml of 0.15M phosphate buffered saline was added and tube labeled and stored at 2° to 8°C, this may be used up to 1 week from time of preparation

**Procedure:**

- (1) For each culture and control, 0.1 ml of 0.1% NBT solution was placed into each well, in a plastic microtiter plate; 6 wells were used, these represent 4 types of examined material, control positive (Histamine) and control negative (Saline).
- (2) Buffy coat cell was collected after centrifugation of heparinized tubes with removal of plasma, and 0.1 ml of this buffy coat was then pipetted into each well. This procedure would increase the concentration of phagocytic cells and was strongly recommended, better than using whole heparinized blood.
- (3) Approximately 0.100 ml of examined materials extract was added to each culture and control wells, labeled as stimulated assay.

- (4) With a plastic pipette mixing was recommended. Then incubation done for 60 minutes, then blood smear was prepared by putting a drop on a microscopic slide.
- (5) For each well of all cultures a blood smear repeated and control.
- (6) One slide selected from each group and flooded with freshly filtered, wright's stain for 3.5 minutes, then in equal volume of Sörenson buffer for 10 minutes more, then washed by distilled water and dried.
- (7) Each slide scored under low power for the greater concentration of leucocytes then, using Fluorescence Microscope, and at least 50 neutrophils were counted, by recording both total neutrophils and the number which contain deposits of black formazone (reduced NBT dye). Only cells containing black material larger than granule, normally appearing in neutrophils, were counted.

The results are expressed as the percent of cells found having phagocytosed, the normal control should have > 25% of the cells exhibiting reduction of NBT dye, the percentage of neutrophils abnormal in NBT if less than 25%.

**Tea Infusion evaluation:**

**Total phenolic content (TPC):**

The TPC was determined as described by Pinelo *et al.* (2005). Total phenolic content was calculated from a standard curve of gallic acid and the results were expressed as milligrams of gallic acid equivalents per gm material individual or their mixture (mg GAE/gm).

**Antioxidant activity (DPPH free radical scavenge):**

The ability of the Red grape pomace extract, black tea, stevioside and formulated tea to scavenge DPPH free radicals was determined by the method described by Blois (1958).

**Sensory evaluation of tea infusions:**

Ten members semi trained panelists from the staff of Food Science Department Ain Shams University were asked to score the formulated tea compared to a normal one for their aroma, flavor, after taste, color and overall acceptability; giving numerical scores to each of their attributes from 10, using a report sheet according to Watts *et al.* (1989).

**Statistical analysis:**

Data were analyzed using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test with  $P \leq 0.05$  being to determine the significant differences in results using SAS software (SAS, 1996).

## **RESULTS AND DISCUSSION**

**Chemical profile of RGP extract:**

**Phenolic compounds:**

The phenolic components of RGP extract was calculated based on calibration curves of external standards built for each of the analyzed compounds. The content of major phenolics detected in the red grape

pomace extract ranged from the highest concentration 54.9 mg/g for catechin to the lowest of 0.26 mg/g for cinnamic as shown in Table (1). These results are in harmony with those obtained by (Murthy *et al.* 2002 & Revilla and Ryan, 2000) stated that the extracts obtained from grape pomace have been used as natural antioxidants due to large quantities of catechins.

**Table 1: Phenolic compounds in RGP extract**

Components	Concentration (mg/g dry matter)
Gallic	2.05
Pyrogallol	24.18
Protocatechuic	8.97
Caffeic	2.21
Vanillic	3.39
Catechin	54.9
Ferulic	3.67
Ellagic	12.11
Cinnamic	0.26
Benzoic	22.97
Syringic	0.68

**Flavonoids compounds:**

The data in Table (2) showed flavonoids contents which were relatively high in red grape pomace extract. It contained relatively high amount of rutin (8.54 mg/g), while the extract had moderate amounts of quercitrin (1.29 mg/g). Minor constituents of quercetin and narenigin (0.24, and 0.16 mg/g, respectively) were observed. While, total flavonoid recorded (3.76 mg/g) as mg quercetin equivalent per gram of RGP extract. These results are in parallel with those of Wang *et al.* (2010) and Rockenbach *et al.* (2011).

**Table 2: Flavonoids compounds in RGP extract**

Components	Concentration (mg/g dry matter)
Rutin	8.54
Quercitrin	1.29
Quercetin	0.24
Narenigin	0.16
Total flavonoid (mg quercetin/g)	3.76

**Phagocytosis inhibition assay:**

Red grape pomace extract, stevioside, black tea and tea infusion formulated was tested against phagocytic function Table (3). Phagocytosis is the first step in the response of macrophages to invading microorganisms. The results showed that each tested material could enhance the ability of uptake of neutral red by macrophages compared with positive control (histamine). Furthermore, red grape pomace extract, stevioside, black tea and formulated tea infusion resulted in a 61, 58, 47 and 60% increase in phagocytosis activity, respectively. These results indicated that the

incorporated black tea with RGP extract and stevioside could activate innate immune response and may be considered as a supplementary therapy.

**Table 3: Immunomodulatory effect of RGP extract, stevioside, black tea and formulated tea infusion on phagocytic response of macrophages**

Treatment groups	Percent phagocytosis
Negative control (saline)	43 ± 1.26 <sup>a</sup>
Positive control (Histamine)	16 ± 0.22 <sup>e</sup>
Red grape pomace extract	41 ± 0.18 <sup>b</sup>
Stevioside	38 ± 0.71 <sup>c</sup>
Black tea	30 ± 0.12 <sup>d</sup>
Tea infusion formulated	40 ± 0.22 <sup>b</sup>

Means having different letters (superscript) in the same column are significantly different (P < 0.05).

**Total phenolic content and scavenging activity of black tea, stevioside and formulated tea:**

The data presented in Table (4) show that RGPE was characterized by high content of total phenols and had a great free radical scavenging activity 78.62%. These results are in agreement with those obtained by Wang *et al.* (2010) and Rockenbach *et al.* (2011). Also, it could be noticed that the formulated tea is a good source of total phenolics content and had a high free radical scavenging activity due to incorporation of red grape pomace extract and stevioside. Therefore, this beverage increases health benefits by increasing antioxidant properties as reported by Robinson *et al.* (1997) and Rao *et al.* (2014).

**Table 4: Total phenolics content and antioxidant activity:**

	Total phenolic (mg GAE/g)	Scavenging activity %
Red grape pomace extract	31.37	78.62
Black tea	12.96	66.23
Stevioside	-	48.15
Formulated tea	46.12	83.25

**Sensory characteristics of tea infusion:**

The mean score values for all parameters of sensory evaluation in formulated tea infusion including: aroma, flavor, after taste, color and overall acceptable are represented in Table (5). Non-significant differences were noticed between tea in the aroma, after taste, color and overall acceptability, while, significant differences were found in the flavor comparing the normal tea which may be the addition of stevioside due to their flavor enhancing properties according to Roberto *et al.* (2012). These results confirm the suitability of red grape pomace extract and stevioside as a supplementation in the production of healthy and simply applicable beverage form in the human nutrition.

**Table 5: Sensory characteristics of tea infusion**

Tea	Aroma	Flavor	After taste	Color	Overall acceptability
Formulated	9.0 ± 0.86 <sup>a</sup>	9.4 ± 0.95 <sup>a</sup>	8.8 ± 0.80 <sup>a</sup>	8.1 ± 0.73 <sup>a</sup>	42.8 ± 0.84 <sup>a</sup>
Normal	8.7 ± 0.63 <sup>a</sup>	7.9 ± 0.94 <sup>b</sup>	8.6 ± 1.20 <sup>a</sup>	7.7 ± 0.63 <sup>a</sup>	39.6 ± 0.75 <sup>a</sup>

Means having different letters (superscript) in the same column are significantly different (P < 0.05).

### **Conclusion**

Based on the above results, it could be concluded that red grape pomace extract and stevioside exhibited a potential source for antioxidant and phytochemical act as immunomodulator effect. They can be incorporated in black tea to enhance their flavor and introduced health promoting beverage.

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نشاط مضادات الأكسدة والتأثير المناعي للشاي الأسود المحتوى على مستخلص  
تفل العنب الأحمر والمحلى بالأستيبيوسيد  
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في هذا البحث تم تحليل مستخلص تفل العنب الأحمر كروماتوجرافياً للمركبات الفينولية ومركبات الفلافونيد وقد تميز المستخلص بمحتواه من الفينولات الكلية وإجمالي مركبات الفلافونيد وكذلك النشاط المضاد للأكسدة في خلب الشقوق الحرة لمركب DPPH وكان إجمالي الفينولات 31.37 ملجم كمكافئ لحمض الجليك / جم مستخلص ، وإجمالي الفلافونيد 3.763 ملجم كمكافئ كيرسيتين / جم مستخلص ، وقد تم تعريف بعض المركبات الفينولية مثل حمض الجليك،البيرجالول،البنزويك، الكاتشين والسيناميك، وإيضاً أظهر تعريف مركبات الفلافونيد كمية عالية من الروتين والكيرسيتين . وقد تم تحضير شاي أسود يحتوى على مستخلص تفل العنب الأحمر ومحلى بالأستيبيوسيد وتقدير النشاط المضاد للأكسدة وإجمالي الفينولات في المستخلص المائي للشاي المحضر والأستيبيوسيد والشاي الأسود للمقارنة . أيضاً في هذه الدراسة تم إجراء إختبار تنبيط الخلايا البالعة لقياس النشاط المحفز للمناعة لكل من مستخلص تفل العنب الأحمر والسنيبيوسيد والشاي الأسود والشاي المحضر وأظهرت الأختبارات المناعية أن المعاملات أظهرت زيادة بلعمة الخلايا البالعة . وإعتماداً على التقييم الحسى أظهر الشاي المحضر قبول عام مقارنة بالشاي العادى ويمكن أن نخلص الى ملاحظة مستخلص تفل العنب الأحمر والأستيبيوسيد بأعتبارهم مكملات في إنتاج مشروبات معززة للصحة.

قام بتحكيم البحث

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