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Research of the antioxidant potential and ability of Black Stuff to scavenge free radicals in vitro

SUMMARY REPORT

2019

Black Stuff powder, a product of Green World Solutions Ltd., has been researched in the RSU Scientific Laboratory of Biochemistry.

RESEARCH TASK

Evaluate the in vitro antioxidant activity of Black Stuff, the product of Green World Solutions Ltd.

The following parameters were used to determine the in vitro antioxidant activity and ability to scavenge free radicals:

- Total Phenolic Content
- DPPH (ability of antioxidants to scavenge 2,2-diphenyl-1-picrylhydrazyl radical)
- FRAP (ability of antioxidants to reduce iron Fe^{3+} to $Fe^{2+)}$
- ABTS (ability of antioxidants to inhibit ABTS cation radical formation). **Experimental part**

The powder was weighed to a concentration of 10 mg/ml and diluted with distilled water. The resulting mixture was diluted with distilled water to a concentration of 5.0; 2.5; 2.0; 1.5; 1.0 and 0.5 mg/ml.

RESULTS

1. Total Phenolic Content

The Cary 50 spectrophotometer (Varian, Inc., the Netherlands) was used for assaying. Reagent Manufacturer: Sigma-Aldrich, Inc. (UNITED STATES).

The content of polyphenols is an important indicator of antioxidant capacity. The method is based on the oxidation of phenol-OH groups in the sample by reaction with Folin-Ciocalteu reagent to form a blue color with an absorption maximum at 765 nm. The color of the solution is directly proportional to the concentration of phenols. The reduction capacity of the Folin-Chicalteu reagent depends on the presence of –OH groups in the polyphenols. Total phenolic compound content was expressed as µg gallic acid equivalent (GAE) per gram of powder using the equation obtained from the gallic acid calibration curve. Three replicates were performed for each sample.

Literature:

1. International organization for standardization – ISO14502-1. Determination of total polyphenols in tea – Colorimetric method using Folin-Ciocalteau reagent. Part I. International organization for Standardization, 2004.

The total phenolic content of the product is shown in Table 1.

Table 1

Total Phenolic Content in Solutions

Koncentration, mg/ml	***µg GAE/g***
1,0	101,2 ± 0,02
0,5	112,4 ± 0,00

The results are the mean of three measurements ± standard deviation

At higher sample concentrations, the phenolic content cannot be determined because the intensity of staining is so high that absorption cannot be determined spectrophotometrically.

2. **Free Radical Scavenging (DPPH) Potential**

The Cary 50 spectrophotometer (Varian, Inc., The Netherlands) was used for assaying. Reagent Manufacturer: Sigma-Aldrich, Inc. (USA).

Stable free radicals, the most widely used of which is 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and which do not exist in vivo, are commonly used as indicators of antioxidant capacity.

DPPH (2,2-diphenyl-1-picrylhydrazil) is a stable organic radical that can be detected by the standard method. During a chemical reaction, a given compound acts as a radical and is a scavenger for antioxidants. The DPPH solution is violet with an absorption maximum at 517 nm. During the reduction, DPPH$^{\bullet}$ scavenges hydrogen from the antioxidant and its color turns yellow. The decrease in absorption intensity is proportional to the antiradical capacity of the natural substances.

The antiradical activity can be judged by comparing the obtained results with the water soluble vitamin E analogue Trolox. The results are expressed as Trolox equivalent (TE) mmol/L and calculated as how many TE equivalents correspond to 1 g of the sample (mmol/g) using the regression equation obtained from the Trolox calibration curve. Three replicates were performed for each sample.

The scavenging potential of the DPPH radical can also be calculated from the degree of inhibition (%) and IC50 (inhibition concentration), which shows the concentration of antioxidant required to achieve a 50% inhibition effect. The IC50 was introduced as a term analogous to "biological" parameters such as LD_{50} (toxicology: lethal dose 50 is the dose causing 50% death in experimental animals). The lower the IC50 value, the higher the antioxidant activity. The DPPH radical scavenging potential can also be calculated from the degree of inhibition (%) using the following formula:

$$I = \frac{(A_0 - A_1)}{A_0} \text{ x } 100\%, \text{ kur} \qquad (1.1)$$

I – *DPPH*• inhibition, %;

A_0 – average absorbance of the 'blank' sample (solvent instead of the sample);

A_1 – mean absorbance of the sample.

Literature:

1. W. Brand – Williams, M. E. Cuvelier and C. Berset "Use of a Free Radical Method to Evalute Antioxidant Activity". Lebensmittel-Wissenschaft & Technologie, 28, 25. – 30. (1995), in *Zürich*.
2. A. Rebiai, T. Lanez and M. L. Belfar., Total Polyphenol Contents, Radical Scavenging and Cyclic Voltammetry of *Algerian Propolis*. Internat. Journal of Pharmacy and Pharmaceutical Sciences., Vol 6(1), 395-400 (2014).

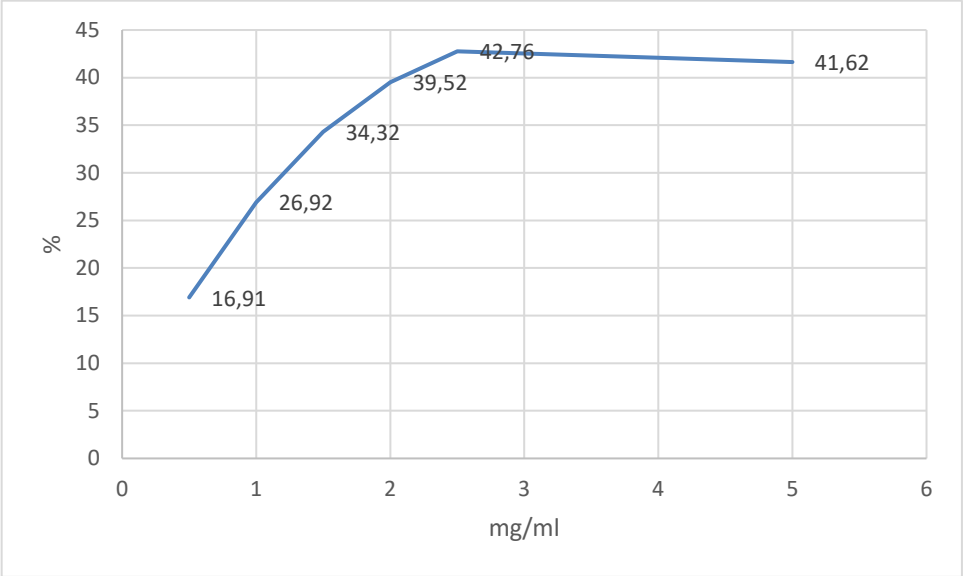
Table 2

Konc. mg/ml	*DPPH•, µmol TE/g*	*DPPH•, µmol TE/L*	*DPPH•, inhibition %*
5	70,60 ± 0,20	353,0 ± 1,4	41,62 ± 0,12
2,5	145,18 ± 1,38	363,0 ± 4,9	42,76 ± 0,40
2,0	167,44 ± 0,15	334,9 ± 0,4	39,52 ± 0,03
1,5	193,19 ± 0,69	289,8 ± 1,5	34,32 ± 0,12
1,0	225,67 ± 4,08	225,7 ± 5,8	26,92 ± 0,47
0,5	277,83 ± 0,17	138,9 ± 0,10	16,91 ± 0,01

DPPH Free Radical Scavenging Potential

The results are the mean of three measurements ± standard deviation

The DPPH inhibition potential of the Black Stuff powder after Trolox is shown in Figure 2.



1. Fig.

Black Stuff powder DPPH radical scavenging potential by degree of inhibition (%)

As shown in Figure 2, the highest inhibitory capacity in aqueous solution was at the concentration of 2.5 mg/ml - 42.8%.

2. **Ferric Ion Reducing Antioxidant Power (FRAP)**

The Cary 50 spectrophotometer (Varian, Inc., The Netherlands) was used for assaying. Reagent Manufacturer: Sigma-Aldrich, Inc. (USA).

By giving away electrons, antioxidants contribute to the reduction of the 3-valent ferric ion to the 2-valent ferric ion in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), producing an intensely blue color with an absorption peak at 593 nm. The decrease in absorbance intensity is directly proportional to the antioxidant capacity of the antioxidants present in the sample. The samples are compared to a standard solution of ferric (Fe^{2+}) sulphate heptahydrate in the aqueous solution.

The results are expressed as Fe^{2+} mmol/L or mmol/100 g using the regression equation obtained from the calibration curve of $FeSO_4 \cdot 7H_2O$. Three replicates were performed for each sample. The higher the antioxidant reduction capacity, the higher the FRAP value.

Literature:

Benzie F. F.; Strain J.J. Ferric Reducing/ Antioxidant power Assay: Direct Measure of Total Antioxidant Activity of biological Fluids and modified Version for Simultaneous Measurement of Total Antioxidant Power and Ascorbic acid Concentration. Methods in Enzymology, vol. 299; 15.–23.; 1999.

Table 3

Black Stuff Ferric Ion Reducing Antioxidant Power (FRAP)

Konc., mg/ml	**mmol Fe^{2+} /100 g**	**mmol Fe^{2+}/L**
10	8,5 ± 0,02	0,846 ± 0,002
5	16,4 ± 0,06	0,822 ± 0,003
2,5	29,2 ± 0,48	0,731 ± 0,012
2,0	35,6 ± 0,05	0,712 ± 0,001
1,5	39,9 ± 0,47	0,598 ±0,007
1,0	52,5 ± 0,02	0,525± 0,016
0,5	62,6 ± 0,01	0,313± 0,004

The results are the mean of three measurements ± standard deviation

Evaluating the ability of bioactive substances in Black Stuff to reduce trivalent ferric ions (Fe^{3+}) to divalent (Fe^{2+}), we can judge their antioxidant capacity.

The obtained FRAP results indicate that the substances contained in Black Stuff have the ability to "donate" an electron to reduce Fe^{3+} to Fe^{2+}, and it is expected that similar compounds may be electron donors to neutralize free radicals.

3. ABTS Radical Cation ($ABTS^{\bullet+}$) Scavenging Capacity

The Cary 50 spectrophotometer (Varian, Inc., The Netherlands) was used for assaying. Reagent Manufacturer: Sigma-Aldrich, Inc. (USA).

The method is based on the formation and inhibition of ABTS cation radicals. The nitrogen atom of ABTS® (2,2-Azino-di- {3-ethylbenzthiazoline sulfonate}) loses its electron by oxidation with potassium persulfate to form $ABTS^{\bullet+}$, which gives a blue-green color to chromophore with an absorption maximum at 734 nm. In the presence of Trolox or other antioxidants, the nitrogen atom scavenges to them with hydrogen and the solution decolorizes. The decrease in absorption intensity is directly proportional to the antioxidant capacity of natural substances.

The results are expressed as Trolox equivalent (TE) mmol/L and calculated as how many TE equivalents correspond to 1 g of the sample (mmol/g) using the regression equation obtained from the Trolox calibration curve. Three replicates were performed for each sample.

The $ABTS^{\bullet+}$ radical scavenging potential can also be calculated from the degree of inhibition (%) as described above (see DPPH detection methods).

Literature

Re, R., N. Pellegrini, A.Proteggente, A. Pannala, M. Yanh, & C.Rice-Evans, 1999, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biology and Medicine, 26 (9-10), 1231-1237.

The determination of the antioxidant capacity of the sample by the ABTS method characterizes the antiradical activity of the antioxidants contained therein, i. that is, the ability to deactivate free radicals by giving them a hydrogen atom. The antiradical activity of the samples is the molar concentration of antioxidants (mmol/L) in which the antioxidants inhibit free radical as a 1 mM Trolox solution. The $ABTS^{\bullet+}$ radical does not exist in vivo.

Table 4

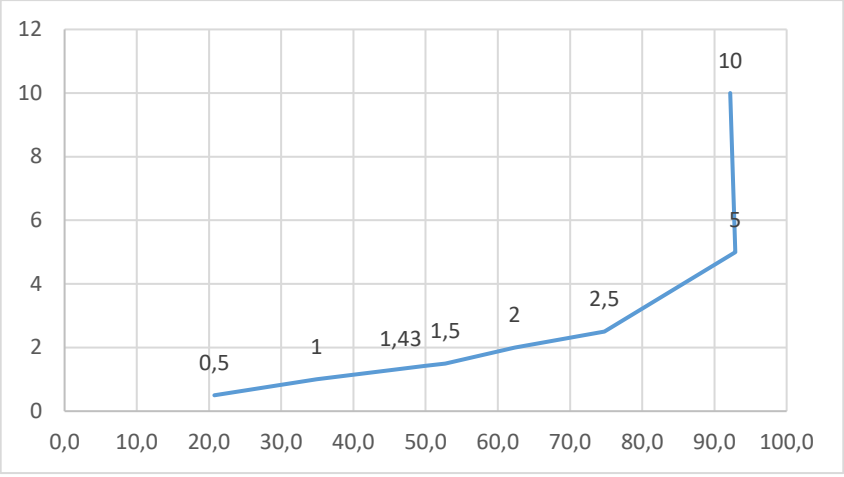
ABTS Radical Scavenging Capacity

Konc., mg/ml	***ABTS mmol TE/g***	***ABTS mmol TE/L***	***ABTS inhibition, %***

10	0,225 ± 0,00	2,25 ± 0,002	92,2 ± 0,1
5	0,454 ± 0,00	2,27 ± 0,003	92,9 ± 0,0
2,5	0,720 ± 0,07	1,80 ± 0,175	74,7 ± 7,4
2,0	0,744 ± 0,10	1,49 ± 0,191	62,3 ± 7,8
1,5	0,835 ± 0,00	1,25 ± 0,005	52,7 ±0,3
1,0	0,805 ± 0,11	0,80 ± 0,107	34,9 ± 4,2
0,5	0,894 ± 0,01	0,45 ± 0,005	20,7 ± 0,1

The results are the mean of three measurements ± standard deviation

The radical inactivation activity, expressed as IC50, shows that Blac Stuff at a concentration of 1.43 mg/ml can halve the initial free radical concentration.



3. Fig. **Black Stuff antioxidant activity in the ABTS assay expressed as IC50**

4. **Total Antioxidant Status**

ABTS® (2,2-Azino-di-{3-ethylbenzthiazoline sulfonate}), when incubated with peroxidase (methmioglobin) and H_2O_2, forms the ABTS® radical cation. It is a relatively stable bluish-green color, measured spectrophotometrically at 600 nm with an RX Daytona analyzer (Randox Laboratories, Ltd., Crumlin, UK) according to the manufacturer's instructions. The antioxidants present in the sample inhibit the formation of this color, which is proportional to its concentration. Reagent Manufacturer: Randox Laboratories, Ltd. (Crumlin, UK). Reagent Kit: Total antioxidant status, Cat. No. NX 2332. Quality Control: Standardized, lyophilized bovine serum - Randox total antioxidant control, Cat. NX 2331. Results are expressed as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent (TE) in mmol/L or mmol/g using the equation obtained from the Trolox calibration curve.

Literature

Miller N.J., Rice-Evans C., Davies M.J., et al. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates // Clinical Science, 1993; 84: 407−412.

The total antioxidant status is shown in Table 5.

Table 5

Total Antioxidant Status

Konc., mg/ml	**mmol TE/g**	**mmol TE/L**
10	0,58	5,83
5	0,84	4,22
2,5	1,02	2,54
2,0	0,98	1,95
1,5	1,22	1,83
1,0	1,34	1,34
0,5	1,36	0,68

Unlike other assays, TAS was determined using a commercially available reagent kit with the RX Daytona Clinical Chemistry Analyzer and one measurement per sample.

Determination of the antioxidant capacity by the TAS method reflects the total molar concentration (mmol/L) of antioxidants compared to the reference standard of the Trolox antioxidant and expressed as its equivalent (TE).

Discussion of results

Antioxidants deactivate radicals through two major mechanisms:

- HAT (Hydrogen Atom Abstraction);
- SET (Single Electron Transfer).

It is very difficult to distinguish between HAT and SET reactions, since both reactions occur simultaneously. The HAT mechanism is used to determine the total antioxidant capacity (ABTS, TAS). The SET mechanism is used to determine the ability of antioxidants to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical and to determine the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} (FRAP).

The antioxidant capacity depends on the presence of bioactive compounds, mainly polyphenols. Polyphenols are known to act as antioxidants by the mechanism of HAT. Comparing the product's antioxidant potentials as determined by TAS, ABTS, FRAP, and DPPH methods, we found that the samples analyzed predominated the antioxidant capacity associated with the HAT mechanism (TAS, ABTS), followed by the FRAP (SET mechanism). DPPH radical scavenging is less pronounced, combining both HAT and SET mechanisms of action.

It is known that antioxidants in natural substances (products) can protect against free radicals, thus reducing or even eliminating the possibility of free radicals damaging cell membranes. The phenols present in this product (Black Stuff) exhibit high antioxidant activity, thus protecting both cell membranes and cell membrane receptors by neutralizing free radicals, while simultaneously regulating the oxidative stress. Hypothetically, prolonged use of Black Stuff improves the body's antioxidant capacity, thereby improving the body's immunity and optimizing other metabolic processes in the body, ensuring Redox homeostasis.

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