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Phenolic Compounds and Biological Activity of Badan (*Bergenia crassifolia* (L.) Fritsch) Leaves Growing in Russia

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ABSTRACT

Objective: Badan or *Bergenia crassifolia* (L.) Fritsch) – is a source of pharmacopoeial CHD – rhizomes of badan (Rhizomata Bergeniae). *B. crassifolia* rhizomes are used in medicine as an astringent, antimicrobial, anti-inflammatory, haemostatic agent. Aqueous and alcohol extracts produced from *B. crassifolia* leaves possess antihypoxic, nootropic, adaptogenic and antioxidant activity. This research aims to carry out comparative study of the composition and content of phenolic compounds (total phenolic glycosides, total flavonoids and total tannins) in the *B. crassifolia* leaves cultivated in the Moscow region (MR) and Tver region (TR) in Russia.

Materials and Methods: 70% ethanol extracts were obtained from air-dry *B. crassifolia* leaves collected in the Tver region (TR) and Moscow region (MR) (Russia).Thin-layer chromatography, spectrophotometry were used to determine total flavonoids content in terms of rutoside, total phenolic glycosides content in terms of arbutin, and permanganatometric titration – total tannins content in terms of tannin. Evaluation of antiradical activity was carried out by a method based on the inhibition of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). To study the membrane-stabilizing and antioxidant activity, a 3% solution of hydrogen peroxide was used, and the antitoxic activity was 2.5% sodium hydroxide solution. 4.5 ml of the culture of ciliates *Paramecium caudatum* in the stationary phase of growth was used.

Results: Rutoside, tannin, gallic acid and arbutin were identified in *B. crassifolia* leaves by TLC.

Total tannins content in TR *B. crassifolia* leaves is 28.21 ± 1.17% and MR *B. crassifolia* leaves – 31.46 ± 1.36%, the total flavonoids content – 2.89 ± 0.11% and 2.39 ± 0.09%, total phenolic glycosides content in terms of arbutin – 19.20 ± 0.37 and 26.04 ± 1.02 % respectively. Antiradical activity of the aqueous-alcoholic badan solution of was confirmed. IC_{50} value of 6.231±0,211 mg/ml was established. The AOA of the initial sample was 23.7%, and for a dilution of 1:10 and 1: 100, it was 91.5% and 83.3%, respectively. This indicates a sufficiently high antiradical activity even with large dilutions of the badan extracts. Badan decoction with the addition of a 3% solution of hydrogen peroxide dilution exhibits antioxidant activity when diluted from 10^{-3} to 10^{-8}. Moreover, the maximum effect is observed at 10^{-4}. Under the toxic effect of 2.5% NaOH solution, the badan decoction increased cell viability at a concentration of 10^{-3}, and the highest value at concentrations of 10^{-5} - 10^{-7}.

Conclusions: The obtained data will be used for development of a pharmacopoeial monograph project "*Bergenia crassifolia* (L.) Fritsch, leaves" for inclusion in the State Pharmacopoeia of the Russian Federation.

Keywords: *Bergenia crassifolia*, thin-layer chromatography, spectrophotometry, flavonoids, tannins, phenolic glycoside

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INTRODUCTION

Vascular brain diseases rank third as cause of death in the statistics of total mortality in Russia. Herbal remedies are used along with synthetic medicines for the treatment and prevention of cerebrovascular accidents. It is known that aqueous and alcohol extracts from the leaves of badan (*Bergenia crassifolia* (L.) Fritsch) possess antihypoxic, nootropic, adaptogenic and antioxidant activity [1-3].

Species that are produced from crud herbal drugs (CHD) with nootropic activity and include the *B. crassifolia* leaves are developed and patented by Pharmacognosy Department employees in cooperation with the Goldberg Research Institute of Pharmacology and Regenerative Medicine employees – Ph.D. Shilova I.V. and Ph.D. Suslov N.I. [1-3].

B. crassifolia – a perennial evergreen plant, is a source of CHD – rhizomes of badan (Rhizomata Bergeniae). *B. crassifolia* rhizomes are used in medicine as an astringent, antimicrobial, anti-inflammatory, haemostatic agent, mainly due to the presence of tannins. According to the published data, a complex of biologically active substances (BAS) of badan leaves consist of phenolic compounds, polysaccharides, etc [4-17].

The aim of the research is a comparative study of the composition and content of phenolic compounds (total phenolic glycosides, total flavonoids and total tannins) in the *B. crassifolia* leaves cultivated in the Moscow and Tver regions (Russia).

MATERIALS AND METHODS

2.1. Plant material

CHD – air-dry *B. crassifolia* leaves – was collected in the Tver region (TR) and Moscow region (MR) (Russia) in 2015-2016 during the growing season.

2.2. Chemicals

ALUGRAM® XtraSILG / UV_{254} 5x7.5 TLC plates were used as stationary phase. Formic acid (chemically pure), ethyl acetate (chemically pure) were purchased from Himmed (Russia). Reference standards (RS) were rutoside (≥97%, CN Acros organics, CAS 153-18-4), arbutin (≥ 98%, Alfa Aesar (Lancaster; Avocado)) and working standard (WS) – tannin and gallic acid.

2.3. Determination of BAS

Studies were conducted at Pharmacognosy Department and Testing Laboratory for the Quality Assurance of Medicines of the Research Institute of Pharmacy.

2.3.1. TLC conditions

The biologically active substances (BAS) composition of *B. crassifolia* leaves was studied by the TLC method. The mobile phase was a water-formic acid-ethyl acetate system (5: 5: 40). Chromatography was carried out in an upward flow of the solvent at a temperature of 20 ° C. The chamber saturation time is 1 hour. Detection was carried out: for all chromatograms in UV light at a wavelength of 254 nm and 365 nm; sequential treatment was applied for phenolic glycosides (10% alcohol sodium hydroxide solution + heating in an oven at a temperature of 100-110°C + diazo-reagent). The following solutions were used in the TLC analysis: water-alcohol extract (70% ethanol) from *B. crassifolia* leaves (TR) - solution No. 1 and *B. crassifolia* leaves (MR) - solution No. 2; solutions of rutoside RS, arbutin RS, tannin WS and gallic acid WS. Test solutions (20 μl) and solutions of rutoside RS (75 μl), tannin WS (15 μl), gallic acid WS and arbutin RS (25 μl each) were applied to TLC plates.

2.3.2. Total tannins, flavonoids, phenolic glycosides content

Determination of total tannins was carried out according to the methodology of SP RF XIII ed., Vol. 2, p. 417 "Determination of the content of tannins in medicinal plant raw materials and herbal preparations" (GPM.1.5.3.0008.15) method 1 (permanganatometric titration). The results of total tannins are expressed in terms of tannin ($C_{76}H_{52}O_{46}$; Mr=1434~1701.18; CAS 1401-55-4) [18]. Determination of the total flavonoids content was carried out by differential spectrophotometry according to the adapted methodology of SP RF XIII ed., Vol. 3, p. 418 "St. John's wort Herb" (PM 2.5.0015.15) [18]. Determination of total phenolic glycosides content was carried out by spectrophotometry method according to the Nagaslayeva L.N. author's technique [19, 20]. The spectrophotometric study was performed at Varian CARY 4000 UV-Vis Spectrophotometer. The wavelength range was from 600 to 200 nm, the cuvette was with a 10 mm layer thickness. CaryWin UV Scan software was used for data processing. Total phenolic glycosides content by Nagaslayeva L.N.: 0.5 g (accurate sample) of the crushed CHD sieved through a laboratory sieve (hole size – 1 mm) was placed in a 250 ml flask, 125 ml of 70% ethanol was added, weighed inaccurately ± 0.01 g, then connected to a reflux condenser and heated in a water bath at 50 ° C for 1 hour with constant stirring. After cooling to room temperature, the flask was weighed and recovered to its original mass by 70% ethanol. The extract was filtered through a cotton swab into a dry flask, discarding the first 15 ml of the filtrate. In parallel with this operation, a column with alumina was prepared. 0.5 cm cotton swab was placed and wetted with water in a 25 cm long, 1.5 cm diameter column (a porous filter No. 2 was soldered into the bottom of the column). 2 g of alumina was placed in a 25 ml beaker, 10 ml of water was added, mixed and poured into a column with an open tap. To the remaining sorbent in the glass, another 10 ml of water was poured, mixed and poured into a column. Above the sorbent, a small cotton swab was placed and washed with 5 ml of 70% ethanol. 2 ml of filtrate was applied to a column filled with alumina and eluted with 25 ml of 70% ethanol at a rate of 4 ml/min. The solution was collected in a 25 ml volumetric flask, adjusted to label by a 70% ethanol and mixed. The column was used once. The resulting eluate was used for analysis, the optical density was determined at 286 nm, 70% ethanol was used as the reference solution.

Determination of the moisture content in *B. crassifolia* CHD was carried out according to the methodology of State Pharmacopoeia of Russian Federation XIII edition, Vol. 2, p. 413 "Determination of the moisture content in medicinal plant material and medicinal herbal preparations" (General Pharmacopoeal Monograph – GPM 1.5.3.0007.15) [18].

2.5. Antiradical activity

Evaluation of antiradical activity was carried out by a method based on the inhibition of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). As a result of the reaction, the intense violet color decreases and the optical density of the DPPH solution also decreases. The reaction mixture consisted of: 2 ml of $5 \cdot 10^{-4}$ M DPPH (Sigma - Aldrich) in 96% ethanol diluted 10 times to obtain a working solution, 2 ml of the test sample (antioxidant). Aqueous-alcoholic extract of badan 70% was used, a series of dilutions was prepared. Samples with DPPH were transferred to a dark place for 30 minutes, after which the optical density was determined by spectrophotometry (at λ = 517 nm). Ethanol served as a comparison solution. The antioxidant activity (AOA) of the test substances was calculated by the formula: AOA = $[(A_0-A_1) / A_0] \times 100\%$, where: A_0 – the antioxidant absorption of the DPPH solution (control); A_1 – the antioxidant activity of the test sample with DPPH. To calculate the IC50 ("Effective Concentration") – the concentration of the substrate, at which 50% of the radicals binds to the test sample, we chose the segment on the straight line plot of the dose-effect graph. The smaller the EC_{50} parameter value, the greater the antiradical activity of a substance [12].

2.6. Assessment of biological activity (membrane-stabilizing, antioxidant and antitoxic activity)

The ability of plant BAS to increase the tolerance of *Paramecium caudatum* Paramecium to cellular poisons may indicate a certain pharmacological effect at the cellular level and in the macroorganism. To study the membrane-stabilizing and antioxidant activity, a 3% solution of hydrogen peroxide was used, and the antitoxic activity was 2.5% sodium hydroxide solution. 4.5 ml of the culture of ciliates *Paramecium caudatum* in the stationary phase of growth was used. In the first test tube, 0.5 ml of distilled water was added and mixed. 0.5 ml of the test sample was added to the second tube, mixed. Then 0.5 ml of liquid was transferred from the second test tube to the third, from the third to the fourth, etc. On a glass slide, three drops containing paramecium (must be at least 5 individuals per drop) were applied. One drop serves as a control; in the second and third drops, 3% H_2O_2 (oxidizing agent), or 2.5% sodium hydroxide solution (toxicant) was added as an unfavorable factor. We observed 5-7 min for a change in the movement of paramecium, noted characteristic changes in their movement: acceleration, deceleration, circular chaotic

movements. With each concentration, the experiments are repeated at least 5 times. Cell death was monitored under a microscope using a stopwatch. The volume leading to 100% cell death within 5 minutes was determined. Further, the index of biological activity of IBA was calculated as the ratio of life expectancy (minutes) under the action of the resolving factor of cells living 24 hours in the medium with the tested concentration of a given object to life expectancy (minutes) under the influence of the irritating factor of cells living 24 hours in the control environment. With a value of the biological activity index of 1.10, an object is considered biologically inactive, with a value of >1.10, the object increases cell viability, with a value of <1.10, the object reduces cell viability [13-15].

Statistical processing of the results was carried out in accordance with the requirements of SP RF XIII GPM.1.1.0013.15 "Statistical processing of experimental results" using the program Microsoft Office Excel 2017 [18].

RESULTS AND DISCUSSION

3.1. Qualitative analysis of phenolic compounds

Chromatographic separation of all *B. crassifolia* CHD alcohol extracts from the in UV light at a wavelength of 254 nm revealed 3 adsorption zones. In the UV light at a wavelength of 365 nm – 2 adsorption zones were detected. Sequential treatment (10% alcohol sodium hydroxide solution + heating in an oven at a temperature of 100-110°C + diazo-reagent) revealed 4 adsorption zones (Table 1).

Table 1: Chromatographic profiles of *B. crassifolia* leaves extracts (70% ethanol)

Standard	UV light, 254 nm (3 adsorption zones)	UV light, 365 nm (2 adsorption zones)	10% alcohol sodium hydroxide solution + heating at 100-110°C + diazo-reagent (4 adsorption zones)
rutoside	Rf = 0.18; green colour	–	Rf = 0,18; yellow color
Arbutin	–	Rf = 0,35; bright blue-violet color	Rf = 0,35; orange-pink color
tannin	Rf = 0.65; dark green color	–	Rf = 0,65; dark brown color
not identified	–	Rf = 0,84; bright red color	–
gallic acid	Rf = 0,88; dark green color	–	Rf = 0,88; brown color

According to the TLC experimental data, rutoside, tannin, gallic acid and arbutin are present in the *B. crassifolia* CHD extracts, it confirms the literature data. The differences in the chromatographic profiles of the *B. crassifolia* leaves extracts, cultivated in TR and MR, were not found.

3.2. Determination of biologically active substances content.

In the course of our study, the total tannin contents, total flavonoids content in terms of rutoside, total phenolic glycosides content in terms of arbutin, and also the moisture content were determined in the *B. crassifolia* leaves.

3.3. Determination of total tannin content

Tannins content can reach up to 27% in *B. crassifolia* leaves according to the literature [3,8].

The results of determining the total tannins content in the test samples are presented in Table 2.

Table 2: Total tannins content in *B. crassifolia* leaves (n = 6, f = 5, P = 95%, T (f, P) = 2.57)

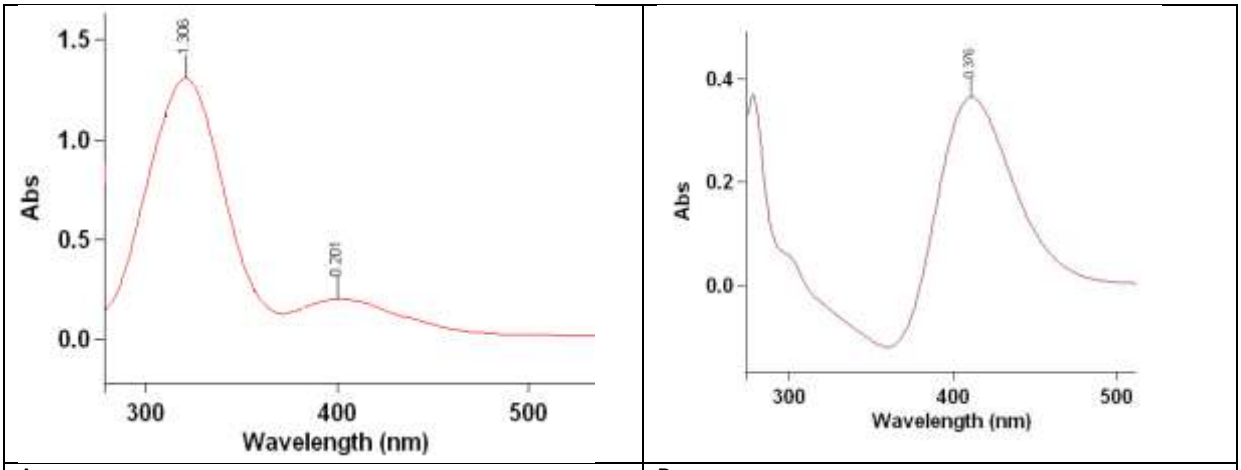
CHD	$\overline{X}$	ΔX	S	E,%
B. crassifolia leaves TR	28,21	1,17	1,116	4,15
B. crassifolia leaves MR	31,46	1,36	1,299	4,33

Note. n – number of repeat tests, f – number of degrees of freedom, P % – confidence figure, $T(f,P)$ – Student's coefficient, $\overline{X}$ – mean value, S – standard deviation, E,% – relative error.

As can be seen from the table 2, total tannins content in *B. crassifolia* leaves in the Tver and Moscow regions is set at 28.21 ± 1.17% and 31.46 ± 1.36%, respectively; in the CHD collected in the MR, total tannins content is insignificantly higher than in the CHD collected in the TR.

3.4. Determination of the content of the amount of total flavonoids content

A study of the spectral characteristics of *B. crassifolia* leaves water-alcohol extracts (70% ethanol) and the spectrum of rutoside RS with aluminum chloride showed their similarity (Fig. 1). It makes possible to recalculate the total flavonoids content in terms of rutoside. The results are shown in Table 3.



A B

Figure 1: Absorption spectrum: A – *B. crassifolia* leaves flavonoid complex with a solution of aluminum chloride; B – rutoside RS with a solution of aluminum chloride

Table 3: The total flavonoids content in *B. crassifolia* leaves (n = 6, f = 5, P = 95%, T (f, P) = 2.57)

CHD	$\overline{X}$	ΔX	S	E,%
B. crassifolia leaves TR	2,89	0,11	0,104	3,79
B. crassifolia leaves MR	2,39	0,09	0,084	3,70

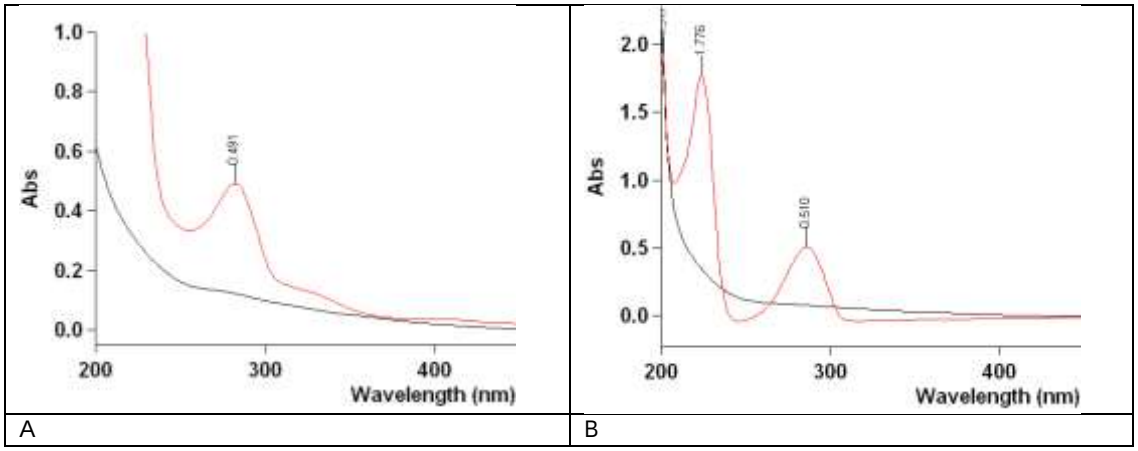
Note. n – number of repeat tests, f – number of degrees of freedom, P % – confidence figure, $T(f,P)$ – Student's coefficient, $\overline{X}$ – mean value, S – standard deviation, E,% – relative error.

According to the obtained data, the total flavonoids content in the *B. crassifolia* leaves TR is 2.89 ± 0.11% in, and *B. crassifolia* leaves MR is slightly less than 2.39 ± 0.09%. This total flavonoids content correlates with literature data [3,8]. It should be noted a higher total flavonoids content in *B. crassifolia* leaves, cultivated in the European part of the Russian Federation, than in CHD collected in Siberia – 1.7% [3].

3.5. Determination of total phenolic glycosides content

Phenolic glycosides are contained (namely, arbutin) in *B. crassifolia* leaves in a significant amount according to the literature data [3, 6, 7, 8]. The presence of arbutin was proved by us in the course of the investigation by TLC.

It has been established that the absorption spectra of water-alcohol *B. crassifolia* leaves TR (Fig. 2 A) and MR extracts (70% ethanol) (Fig. 2 B) are identical and have a maximum at a wavelength of 286 nm (after preliminary purification on an alumina column).



A B

Figure 2: Absorption spectrum: A – purified *B. crassifolia* leaves water-alcohol extract; B – arbutin RS water-alcohol solution

Determination of total phenolic glycosides content in terms of arbutin in CHD was calculated by the formula:

$$X, \% = \frac{D \cdot 100 \cdot 25 \cdot K}{E_{1см}^{1\%} \cdot m \cdot a},$$

where K = 1,14025 is the coefficient of incomplete elution [16]

$E_{1см}^{1\%}$ = 72.23 – the specific absorption index

m – mass with taken into account humidity, g

a – an aliquot, ml

Table 4: The total phenolic glycosides content in terms of arbutin in *B. crassifolia* leaves (n = 6, f = 5, P = 95%, T (f, P) = 2.57)

CHD	$\overline{X}$	ΔX	S	E,%
B. crassifolia leaves TR	19,20	0,74	0,714	3,90
B. crassifolia leaves MR	26,03	0,96	0,916	3,69

Note. n – number of repeat tests, f – number of degrees of freedom, P % – confidence figure, $T(f,P)$ – Student's coefficient, $\overline{X}$ – mean value, S – standard deviation, E,% – relative error.

The results of determining the total phenolic glycosides content in terms of arbutin in *B. crassifolia* leaves are shown in Table 4. From the results presented in Table 4, it can be seen that total phenolic glycosides content in terms of arbutin in *B. crassifolia* leaves collected in TR and MR was, respectively, 19.20 ± 0.37 and 26.04 ± 1.02 %. It is obvious that in *B. crassifolia* leaves MR of phenolic glycosides accumulates by almost 30% more, but, in general, the content of phenolic glycosides is confirmed by the literature data [3, 6, 8].

3.6. Antiradical activity

Antiradical activity of the aqueous-alcoholic solution of badan was confirmed. This is due to the content of natural antioxidants, namely phenolic compounds. IC_{50} value of 6.231±0,211 mg/ml was established. The AOA of the initial sample was 23.7%, and for a dilution of 1:10 and 1: 100, it was 91.5% and 83.3%, respectively. This indicates a sufficiently high antiradical activity even with large dilutions of the badan extracts.

3.7. Assessment of biological activity (membrane-stabilizing, antioxidant and antitoxic activity)

Badan decoction with the addition of a 3% solution of hydrogen peroxide dilution exhibits antioxidant activity when diluted from 10^{-3} to 10^{-8}. Moreover, the maximum effect is observed at 10^{-4}. Under the toxic effect of 2.5% NaOH solution, the badan decoction increased cell viability at a concentration of 10^{-3}, and the highest value at concentrations of 10^{-5} - 10^{-7} (Table 5, Figure 3).

Table 5: Evaluation of the membrane-stabilizing, antioxidant and antitoxic effects of badan decoction

No of experiment	Degree of dilution of badan decoction	Biological activity index (BAI) * 3% H_2O_2	Biological activity index (BAI) * NaOH 2.5%
1	1	-	-
2	10^{-1}	-	-
3	10^{-2}	0,58	1,07
4	10^{-3}	1,49	1,22
5	10^{-4}	2,73	0,76
6	10^{-5}	2,21	2,84
7	10^{-6}	1,69	2,67
8	10^{-7}	1,66	2,84
9	10^{-8}	1,36	0,82
10	10^{-9}	0,78	0,93

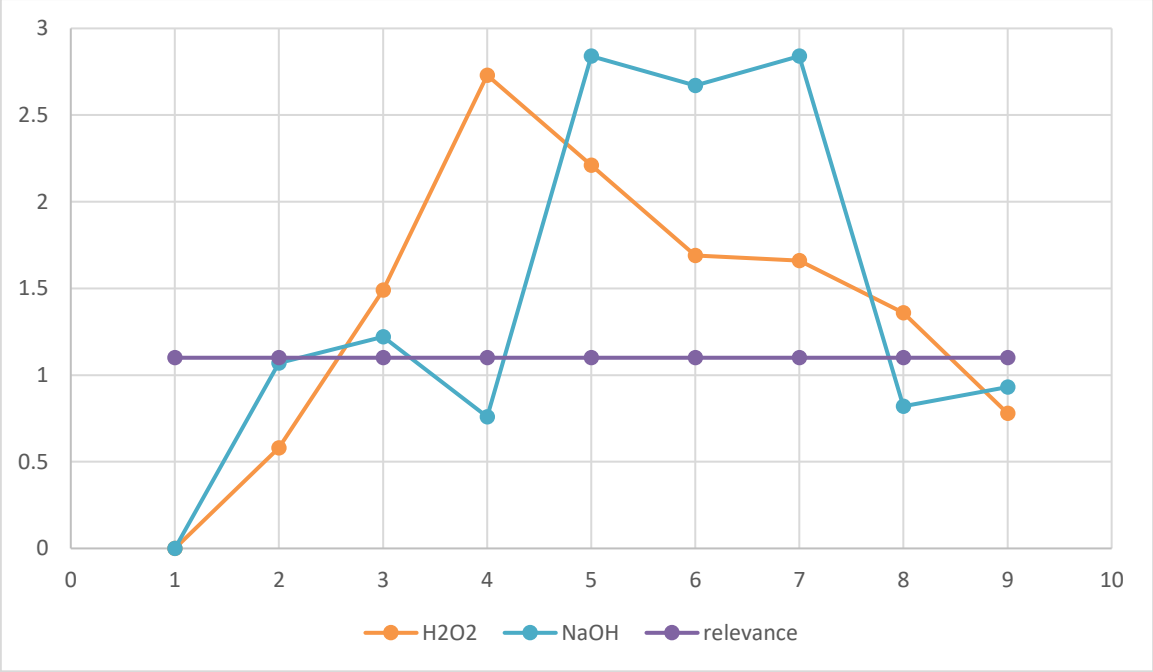


Figure 3: Membrane-stabilizing, antioxidant and antitoxic effects of badan decoction.

CONCLUSION

1. The identity of the phenolic compounds composition of *B. crassifolia* leaves harvested in the MR and TR was established using the thin-layer chromatography method
2. Rutoside, tannin, gallic acid and arbutin were identified in the studied *B. crassifolia* leaves extracts when compared with reference and working standards
3. Quantitative analysis of the biologically active substances of MR and TR *B. crassifolia* leaves showed similar results.
4. The obtained data will be used in the development of a draft pharmacopoeial monograph on *B. crassifolia* leaves.

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