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CORRELATION BETWEEN THE ESTIMATED TOTAL THIOSULFINATES CONTENT AND ANTIPLATELET ACTIVITY OF THREE DIFFERENT VARIETIES *A. CEPA*

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ABSTRACT. The present study aims to establish a correlation between the estimated thiosulfinate compound content of *Allium cepa L. (A. cepa)* juices and their antiplatelet activity. The juices were obtained from three different varieties of *A. cepa,* cultivated in three different regions of Romania.

The thiosulfinate compound content was estimated using a spectrophotometric method, based on the reaction with 4-mercapto-pyridine (a chromogenic thiol, with a maximum absorbance coefficient at 324 nm). The constant of the reaction kinetic curve was obtained by overlapping experimental data with an exponential function of first degree.

The antiplatelet activity of the mentioned juices was measured by using *in vitro* tests with platelet rich plasma (PRP) obtained from blood collected from healthy human people, with arachidonic acid as platelet agonist.

A statistically significant direct proportionality between the estimated thiosulfinate compound content and the antiplatelet activity of the tested *A.cepa* juices was established.

Keywords: *Allium cepa; natural products; platelet; antiplatelet; thiosulfinate compounds.*

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INTRODUCTION

Allium genus, with over 500 species, belongs to the family *Amaryllidaceae (Alliaceae)*, subfamily *Allioideae. Allium cepa* (*A. cepa*) is a biennial plant that produces a bulb in the first year [1-3].

Water content represents 80-95% from the weight of the fresh onion, the rest of 5-20% being represented by dried substance. From the last one, over 65% was found like non-structural carbohydrates [4]. Other categories of chemical species such as flavonoids and organo-sulfur compounds, with beneficial effects on human health, were identified in the dried substance.

Organosulfur compounds are represented by: non-volatile S-amino acids, derivatives of cysteine, S-alk(en)nyl-L-cysteine sulfoxides (ACSOs) and their degradation products: thiosulfinate compounds and poly-sulfides. The ACSOs are the ones responsible for the characteristic odor, that becomes manifest at the cleavage in the presence of alliinase (alliin alkylsulphenate-lyase). ACSOs generate the characteristic odor and taste. Some of the therapeutic effects of the *A. cepa* are due to the sulfur compounds, formed by cleavage of three types of S-alk(en)nyl-L-cysteine sulfoxides (ACSOs) in the presence of alliinase [5]. In the intact tissue, ACSOs and alliinase are stored in different cellular compartments. Injury of the tissue, that is the destruction of these compartments, takes to ACSO hydrolysis. Consequently, iminopropionic and S-alk(en)yl-cystein-sulphenic acids are formed in the presence of alliinase.

There are many studies focused on the antiplatelet activity of the *A. cepa* juices as well as on their anti-atherosclerotic effects and alteration of the serum lipid profile [6]. Studies on the antiplatelet activity of the aqueous extract of onion suggest the inhibition of the arachidonic acid release from phospholipids, the process that initiates the eicosanoid metabolism leading to the synthesis of prostaglandins, thromboxanes and leucotrienes [7]. Thiosulfinate compounds of *A. cepa* seem to be the active constituents with antiplatelet activity, *via* their inhibition effect on the COX activity, including the arachidonic acid metabolism and the formation of TxA_2 [8].

Because the majority of the studies sustain that the mechanism of the antiplatelet activity of the *A. cepa* juices is based on the COX activity inhibition, one of the platelet agonists chosen to be used in our study is the arachidonic acid.

Many methods have been described for the identification of thiosulfinate compounds from *A. cepa* juices or extracts: fast spectrophotometric determination [9], the use of HPLC (with a chiral stationary phase for the separation of the thiosufinate esters from natural/synthetic extracts of *A. cepa* [10]), H-NMR [11] or GC-MS [12]. Combined analytical methods are also reported. Other

determination methods for the alliicin and alliinase activity include the reaction between 2-nitro-5-tiobenzoat (NTB) and alliicin [13] or that of thiosulfinate compounds with chromogenic thiols like mercaptopyridine (2-MP), 4-mercapto-pyridine (4-MP), 1-oxide-2-mercaptopyridine (MPO) and 2-mercaptopyrimidine (MPM), respectively [14].

The aim of this study was to correlate the thiosulfinate compound content from *A. cepa* juices obtained from the three studied varieties with their tested antiplatelet activity by using *in vitro* tests on platelet rich plasma (PRP).

Since most of the studies are focused on examining the effect and the mechanism of platelet aggregation inhibition or on the determination of the relative concentration of the extracts/juices [15-17], this study represents a novelty.

RESULTS AND DISCUSSION

The percentage of the recovered juice from the studied varieties of *A. cepa*, reveals a higher value for the yellow varieties as compared to the white one (figure 1). Some studies are in agreement with our results [18], some on the contrary [19], which shows the existence of multiple variables (such as: raw material, the process used) that can influence the process.

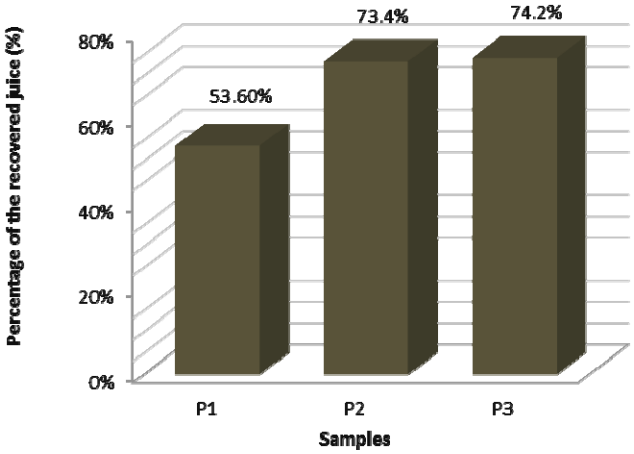


Figure 1. The percentage of the recovered juice *(defined as the ratio between the amount of the juice and the amount of raw material of A. cepa)*

Determination of the total thiosulfinate content

The reaction with 4-mercapto-pyridine (4MP) was used to estimate the thiosulfinate content from *Allium cepa* juice. 4-MP is a chromogenic thiol, commercialized in pure form. It is a stable, inert compound, which reacts with the thiosulfinate compounds from *Allium* juice (alliicin, alliin) [14, 20]. 4-MP presents an absorbance maximum at 324nm wavelength (ε = 19,600 $M^{-1}cm^{-1}$).

The kinetic method for the determination of tiosulfinate content (aliicin, alliin) is temperature and pH dependent [21].

We defined the kinetic curve obtained for sample 1 as a calibration curve in order to estimate the thiosulfinate content from the juices of A. *cepa* (figure 2 left). The highest quantity of 4-MP was used during the reaction with the sample 1. Thiosulfinate compounds from *A. cepa* juices were determined in relation to the reaction kinetic constant of the kinetic curve, an exponential function of first degree [22].

In figure 2left depicts the kinetic curve of the consumption of 4-MP during the reaction with thiosulfinates of juice obtained from sample 1 - the white variety of *A. cepa* (the kinetic constant of the reaction is calculated in function of this). Figure 2 right shows superior estimated amounts of thiosulfinate compounds in the white *A. cepa* than in the other studied yellow varieties.

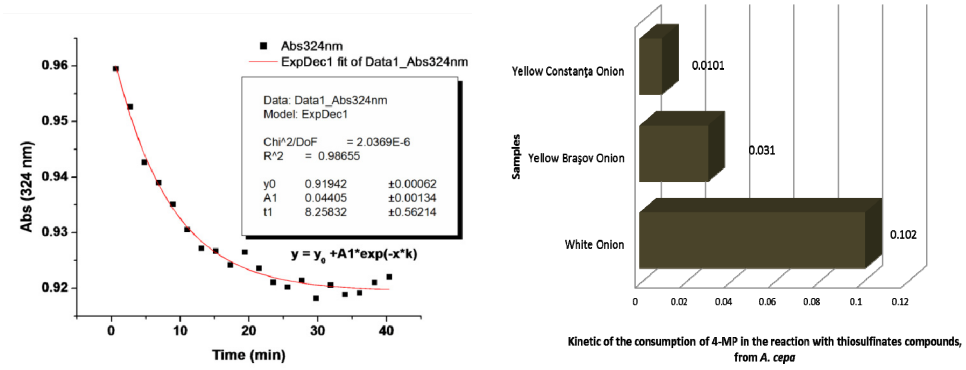


Figure 2. left) Bleaching of 4-MP by thiosulfinates in sample 1; **right)** Kinetics of the consumption of 4-MP in the reaction with tiosulfinate compounds, (**x-axis** = sample; **y-axis** = coefficient of kinetic consumption (k_1 - min^{-1}))

Determination of antiplatelet activity

We quantified the antiplatelet activity by *in vitro* tests using PRP obtained from blood collected from healthy humans. The principle of the method is based on the increase of the transmittance during the aggregation process. This was recorded by using a spectrophotometer (with magnetic stirrer) at 600 nm wavelenght.

During the antiplatelet effect testing procedure, a significant increase of transmittance was observed for the control sample, in the same time with platelet aggregation. Hence an obvious inhibition of platelet aggregation in the presence of *A. cepa* juice was sensed.

The percentage of the inhibition of platelet aggregation in the presence of *A. cepa* juice was calculated with respect to the maximum transmittance at 7 min (considered the final point of the platelet aggregation inhibition reaction) for the test sample and for the control sample like in figure 3 left, like an extrapolation of our previous researches (in press) [22]. It was observed that sample 1, the white *A. cepa* variety, has the strongest antiplatelet effect, with an inhibition percentage of 87.2% and SD of 2.3% (see figure 3 right).

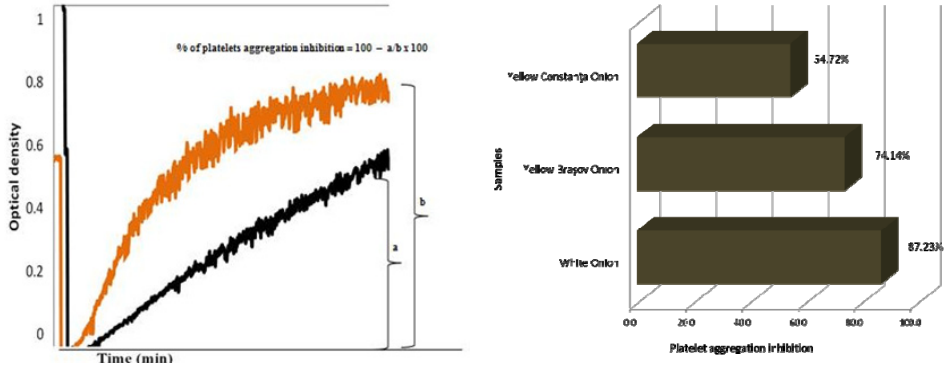


Figure 3. left) Method for calculation of the antiplatelet activity (**a** = maximum transmittance at 7 min for test sample; **b** = maximum transmittance at 7 min for control sample); **right)** The inhibition of the platelet aggregation in the presence of the *A. cepa* juices (**x-axis** = sample; **y-axis** = percentage of the platelet aggregation inhibition).

Thiosulfinate compounds are considered to be responsible for the antiplatelet activity [16, 23]. For this purpose, we have determined the correlation between the estimated thiosulfinate compound content of the studied juices and their effect of platelet aggregation inhibition. (Table 1) This is in agreement with the literature data.

After applying the statistical test described in the „Experimental section" a *Pearson correlation coefficient* of (*r)* = 0.914 was obtained. It indicates a strong positive correlation between the two sets of values. The *p-value* of 0.0167, obtained by applying the „*T-test*", showed that the correlation is statistically significant.

Table 1. The percentage of the recovered juice, coefficient of kinetic consumption of 4-MP in the reaction with thiosulfinate compounds and percentage of platelet aggregation inhibition for the three different varieties of *A. cepa*

Allium cepa Variety	Recovered juice (%)	Coefficient of kinetic consumption of 4-MP by thiosulfinate compounds (k_1 - min^{-1})	Percentage of platelet aggregation inhibition (%)	Standard Deviation (%)
P1 - White *A. cepa*	53.6	0.1020	87.2	2.3
P2 - Yellow *A. cepa* (Braşov)	73.4	0.0310	74.1	2.4
P3 - Yellow *A. cepa* (Constanţa)	74.2	0.0101	54.7	2.3

Because there is no literature data concerning the comparation of the antiplatelet activity of different varieties of *Allium cepa*, the authors focused their studies on this part as well [22].

CONCLUSIONS

A direct proportionality between the estimated thiosulfinate compound content and the antiplatelet activity of the tested A. cepa juices was observed, but further researches in this direction are needed. The juice of white A. *cepa* has higher antiplatelet activity as well as estimated quantity of thiosulfinate compound than the yellow *A. cepa*.

EXPERIMENTAL SECTION

Obtaining the *Allium cepa* juice

Three varieties of *A. cepa* were used, grown in three different regions of Romania, approximately in the same period of the year, treated similarly against diseases and pests (Table 1). The juice was obtained from portioned *A. cepa* bulbs using an electrical juicer. It was further centrifuged for about 20 minutes at 10000rpm with the recovery of the supernatant.

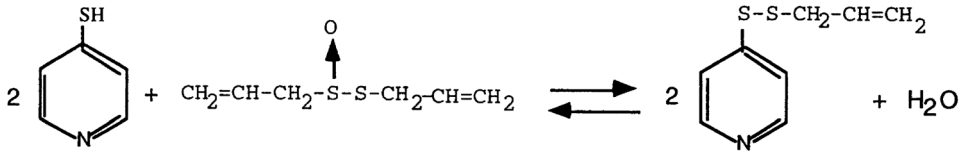
Determination of the total thiosulfinate compound content

A buffer solution of 4-MP, with a pH=7.2, was prepared in order to estimate the thiosulfinate compound content. The principle of the method is based on the reaction of 4-MP with the activated disulfide bound from the thiosulfide compund -S(O)-S-. It determines the consumption of 4-MP during the chemical reaction with formation of mixed disulfides, 4-allyl-mercapto-pyridine that do not absorbe at 324 nm wavelength [13, 20].

The consumption rate of 4-MP during the reaction was monitored by spectrophotometric means. The continuous measurement of the optical density (OD) during the reaction between 4-MP and the juice of *A. cepa*, permitted the recording of the absorbance decrease during time at the specified wavelength (concomitantly with the consumption of 4-MP).

Determination of the kinetic constant of the reaction is absolutely necessary [20]. This parameter was calculated at 24˚C and pH=7.2 for each sample, by using a first order exponential decay function (figure 2left).

After adding a volume of the *A. cepa* juice to the solution of 4-MP, absorbance of the samples was recorded every 2 minutes, during 40 minutes, at room temperature by using a spectrophotometer (UNICAM 4, UV-Vis spectrophotometer).



Scheme 1 [20]

Determination of antiplatelet activity

The calibration of the spectrophotometer was done with platelet poor plasma (PPP) and PRP, considered to have transmittance values of 100% and 0%, respectively.

To the PRP sample a well defined antiplatelet agonist (**Arahidonic Acid**, concentration 0.685mM-Sigma-Aldrich, from porcine liver, BioReagent, suitable for cell culture,>99%, USA) was added. For the control and for the test samples first a preset quantity (1:100; v:v) *A. cepa* juice was and then the antiplatelet agonist under continuous recording of transmittance until 7 minutes.

The antiplatelet agonist was added by pipetting it directly into the PRP, not in a part of the cuvette, with the aim to avoid the formation of air bubbles. Two sets of analyses were carried out for the control samples and five sets for the test samples for each variety of *A. cepa*. Presented results are averages of all obtained values.

Statistical test: The “Pearson Correlation” was used for comparison between the means of two quantitative variables. The correlation coefficient r measures the strength and direction of a linear relationship between two variables; the value of r is always between (+1) and (-1). A correlation coefficient of (+1) indicates a perfect positive correlation; (-1) - indicates a perfect negative correlation; near 0 - indicates no correlation.

A “T-test” was used to determine whether the correlation coefficient is “strong” or “significant” or not. It is considered statistically significant when the p value is under 0.05 and statistically highly significant when lower than 0.001.

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