IN VITRO SCREENING OF CRATAEGUS SUCCULENTA EXTRACTS FOR FREE RADICAL SCAVENGING AND 15-LIPOXYGENASE INHIBITORY ACTIVITIES

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IN VITRO SCREENING OF CRATAEGUS SUCCULENTA EXTRACTS FOR FREE RADICAL SCAVENGING AND 15-LIPOXYGENASE INHIBITORY ACTIVITIES (Abstract): Crataegus succulenta Schrad. ex Link is widely spread in North America. A literature survey revealed no studies on the chemical composition and biological effects of this species. Aim: The aim of the present study was to investigate the phenolic content, free radical scavenging and 15-lipoxygenase inhibitory effects of Crataegus succulenta leaf and flower extracts. Material and methods: Total phenolic, flavonoid and proanthocyanidin contents were quantified by spectrophotometric methods. Both extracts were evaluated for their ability to scavenge DPPH, superoxide anion and hydroxyl radicals and to inhibit 15-lipoxygenase activity. Results: There were noticed no striking differences in the total phenolic, flavonoid and proanthocyanidin contents between leaf and flower extracts. Both extracts showed similar 15-lipoxygenase inhibitory effects. Flower extract scavenged more effectively DPPH and superoxide radicals while leaf extract was more active against hydroxyl radical. In superoxide anion radical scavenging assay, both extracts were more active than (+)-catechin. In hydroxyl radical scavenging and 15-lipoxygenase inhibition assays, the extracts were only 4-5 times less active than (+)-catechin. Conclusions: The high antioxidant potential of Crataegus succulenta extracts suggest a possible use as ingredients in functional foods for the prevention of oxidative stress-related diseases. Keywords: CRATAEGUS SUCCULENTA, SUPEROXIDE ANION RADICAL, HYDROXYL RADICAL, 15-LIPOXYGENASE

The genus Crataegus (Rosaceae) consists of approximately 300 species widely spread in the northern hemisphere. Many of these species have been used in folk medicine in the treatment of gastrointestinal, urinary and cardiovascular disorders. Flavonoids (vitexin, orientin and their derivatives, hyperoside, rutin) and oligomeric procyanidins are the main phytochemicals that have been identified in the leaves, flowers and berries of many Crataegus species. Crataegus extracts have beneficial effects on the cardiovascular system exhibiting antioxidant, antiatherosclerotic, hypo-
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tensive, positive inotropic, coronary dilatation and antiarrhythmic effects (1, 2).

*Crataegus succulenta* Schrad. ex Link (syn. *Crataegus macracantha* Loudon, fleshy hawthorn, succulent hawthorn) is widely spread in North America (3). The species has not been studied regarding its chemical composition and possible biological effects. In this respect, the aim of the present work was to investigate the phenolic content, free radical scavenging and 15-lipoxygenase inhibitory effects of leaf and flower extracts.

**MATERIAL AND METHODS**

**Chemicals.**

Tris(hydroxymethyl)aminomethane (Tris) and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), pyrogallol, Folin-Ciocalteu's phenol reagent, linoleic acid, lipoxydase from soybean were from Fluka (Steinheim, Germany). (+)-Catechin hydrate, gallic acid, sodium carbonate, 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical, iron (II) sulfate heptahydrate, hydrogen peroxide, sodium salicylate were from Sigma-Aldrich (Steinheim, Germany). Ammonium iron (III) sulfate dodecahydrate and sodium nitrite were obtained from Riedel-de Haën (Seelze, Germany). All other solvents and reagents were of analytical grade.

**Plant material.** Leaves and flowers of *Crataegus succulenta* Schrad. ex Link were collected in the Botanical Garden "Anastasie Fatu", Iasi in May 2010. The identity of the plant material was confirmed by taxonomists from Botanical Garden "Anastasie Fatu". Leaves and flowers were dried in dark at room temperature. Voucher specimens have been deposited in the Discipline of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa"-Iasi.

**Extraction.** Dried and powdered leaves and flowers (50 g each) were extracted with 500 mL of 70% aqueous methanol by stirring for 6 h at room temperature. The extraction was repeated twice. The combined extracts were concentrated under reduced pressure at 40°C and then freeze-dried.

**Total phenolic content.** Total phenolic content was quantified by Folin-Ciocalteu method as previously described (4). Electron-donating compounds such as phenols react with phosphomolybdic/ phosphotungstic acid complexes in Folin-Ciocalteu reagent and form a blue complex. The results were expressed as g gallic acid equivalents (GAE)/100 g extract using the following equation based on the calibration curve: y=0.1165x+0.0269, R²=0.9987.

**Flavonoid content.** Flavonoids were quantified by a spectrophotometric method based on their ability to generate pink coloured products in the presence of sodium nitrite and aluminium chloride, in alkaline solution (5). The results were expressed as g catechin equivalents/100 g extract using the following equation based on the calibration curve: y=0.0759x - 0.0038, R²=0.9984.

**Proanthocyanidin content.** The assay, described by Porter et al., is based on the conversion of proanthocyanidins to anthocyanidins in n-butanol-HCl and in the presence of ferric salts (6). The results were expressed as cyanidin equivalents/100 g extract using the molar extinction coefficient and molecular mass of cyanidin (ε=17,360 L·mol⁻¹·cm⁻¹ and M=287.24, respectively) (7).

**DPPH radical scavenging assay.** Antioxidants reduce DPPH radical (purple) to diphenylpicrylhydrazine DPPH-H (yellow) leading to a decrease in absorbance at 517
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nm (4). (+)-Catechin hydrate was used as positive control. DPPH scavenging activity (%) was calculated as:

\[ 100 \times \left( \frac{A_{\text{start}} - A_{\text{end}}}{A_{\text{start}}} \right) \]

where \(A_{\text{start}}\) and \(A_{\text{end}}\) are the absorbances of DPPH radical solution before and 5 min after adding the samples, respectively.

**Superoxide anion radical scavenging assay.** Superoxide anion radical, generated by the auto-oxidation of pyrogallol, oxidizes the latter to quinones that are quantified spectrophotometrically (8). (+)-Catechin hydrate was used as positive control. Superoxide anion radical scavenging activity (%) was calculated as follows: 100 \times \left( \frac{\text{slope}_{\text{control}} - \text{slope}_{\text{sample}}}{\text{slope}_{\text{control}}} \right) \] where \(\text{slope}_{\text{control}}\) and \(\text{slope}_{\text{sample}}\) are the slopes of the plots of absorbance vs. time for control and samples, respectively.

**Hydroxyl radical scavenging assay.** The assay is based on the ability of hydroxyl radical, generated by Fenton reaction, to produce a violet product in the presence of sodium salicylate and ferric ions (9). (+)-Catechin hydrate was the positive control. Hydroxyl radical scavenging activity (%) was calculated as: 100 \times \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \] where \(A_{\text{control}}\) is the absorbance of the control and \(A_{\text{sample}}\) is the absorbance in the presence of extracts/(+)-catechin.

**15-Lipoxygenase inhibition assay.** In the presence of 15-lipoxygenase, linoleic acid is oxidized to conjugated dienes which strongly absorb at 234 nm. The assay was performed as described by Wangensteen et al. with minor changes (4). (+)-Catechin hydrate was used as positive control. 15-Lipoxygenase inhibition (%) was determined on the basis of the absorbance of the control and samples after 30 and 90 s reaction time as follows:

\[ 100 \times \left\{ \left( \frac{A_{\text{control},90} - A_{\text{control},30}}{A_{\text{control},90}} \right) - \left( \frac{A_{\text{sample},90} - A_{\text{sample},30}}{A_{\text{control},90}} \right) \right\} \]

**Statistical analysis.** All experiments were carried out in triplicate. The results were expressed as means±standard deviations. The EC\(_{50}\) values were calculated by linear interpolation between values above and below 50% activity.

**RESULTS**

**Extraction.** The extraction afforded 13.3 g of leaf extract (S-l, yield: 26.6%) and 13.1 g of flower extract (S-f, yield: 26.2%).

**Total phenolic, flavonoid and proanthocyanidin contents.** S-l and S-f contained similar amounts of total phenolics, flavonoids and proanthocyanidins (tab. I).

<table>
<thead>
<tr>
<th>Extract</th>
<th>Total phenolic content (%)</th>
<th>Flavonoid content (%)</th>
<th>Proanthocyanidin content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-l</td>
<td>15.33 ± 0.40</td>
<td>5.25 ± 0.42</td>
<td>3.5 ± 0.0</td>
</tr>
<tr>
<td>S-f</td>
<td>16.15 ± 0.23</td>
<td>5.16 ± 0.30</td>
<td>3.65 ± 0.17</td>
</tr>
</tbody>
</table>

S-l – leaf extract; S-f – flower extract

**DPPH radical scavenging assay.** DPPH radical scavenging effects of S-l and S-f were found to increase in a dose-dependent manner from 11.96±0.14 and 13.31±0.24%, respectively at 10.41 μg/mL to 89.08±0.31 and 92.62±0.04%, respectively at 166.66 μg/mL. At the same concentrations, (+)-catechin exhibited scavenging effects of
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84.49±0.50 and 95.37±0.47%, respectively (fig. 1). An additional dilution (5.20 µg/mL) was tested in order to determine the EC$_{50}$ value of (+)-catechin; at this dilution, a scavenging activity of 46.98±0.11% was registered. According to the EC$_{50}$ values, S-f (46.7±0.2 µg/mL) was more active than S-l (52.0±0.5 µg/mL). The EC$_{50}$ value of (+)-catechin was found to be 5.5±0.0 µg/mL suggesting a higher DPPH radical scavenging ability than Crataegus succulenta extracts (tab. II).

**Fig. 1.** DPPH radical scavenging activity of *Crataegus succulenta* extracts (S-l – leaf extract; S-f – flower extract; Cat – (+)-catechin)

Superoxide anion radical scavenging assay. Superoxide anion radical scavenging activity was dose dependent for both extracts. In the concentration range of 0.65-0.87 mg/mL, (+)-catechin exhibited only 20.15-24.02% scavenging activity (fig. 2). Therefore, in order to calculate the EC$_{50}$ value of (+)-catechin, higher concentrations (1.61, 2.26 and 2.58 mg/mL) were tested. At these concentrations, (+)-catechin scavenged superoxide anion radical by 40.15±1.31, 48!83±0.00 and 52.70±1.33%, respectively. Within this assay, both extracts were more active than (+)-catechin; the EC$_{50}$ value of S-f (0.65±0.01 mg/mL) was lower than that of S-l (0.73±0.00 mg/mL) suggesting stronger scavenging properties for the former (tab. II).

Hydroxyl radical scavenging assay. In this assay, both extracts showed concentration-dependent scavenging effects but they were less active than (+)-catechin. At 1.87 mg/mL, the scavenging effects of S-l, S-f and (+)-catechin were found to be 56.57±0.79, 66.76±0.80 and 90.01±1.81%, respectively (fig. 3). With regard to the EC$_{50}$ values, S-l (1.26±0.05 mg/mL) and S-f (1.6±0.0 mg/mL) were only 4-5 times less active than (+)-catechin (0.3±0.0 mg/mL) (tab. II).

15-Lipoxygenase inhibition assay. *Crataegus succulenta* extracts inhibited 15-lipoxygenase in a concentration-dependent manner with similar EC$_{50}$ values (S-l: 154.6±0.6 µg/mL, S-f: 154.86±0.35 µg/mL) (fig. 4, tab. II). In the range of tested concentrations (100-166.66 µg/mL), (+)-catechin exhibited high activity (95.22±0.76-99.99±0.01%); therefore, lower concentrations (20.83, 41.66 and 83.33 µg/mL) were tested in order to determine its EC$_{50}$ value; at these concentrations, catechin exhibited 31.65±0.53, 67.05±0.16 and 87.07±0.26% scavenging activity, respectively. (+)-Catechin inhibited 15-
lipooxygenase activity with an EC\textsubscript{50} value of 29.2±0.2 µg/mL. With regard to the EC\textsubscript{50} values, both extracts were 5 times less active than (+)-catechin (tab. II).

**Fig. 2.** Superoxide anion radical scavenging activity of *Crataegus succulenta* extracts (S-l – leave extract; S-f – flower extract; Cat – (+)-catechin)

**Fig. 3.** Hydroxyl radical scavenging activity of *Crataegus succulenta* extracts (S-l – leave extract; S-f – flower extract; Cat – (+)-catechin)

**Fig. 4.** 15-Lipoxygenase inhibitory activity of *Crataegus succulenta* extracts (S-l – leave extract; S-f – flower extract; Cat – (+)-catechin)
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**TABLE II**

Free radical scavenging and 15-lipoxygenase inhibitory activities of *Crataegus succulenta* extracts

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>DPPH radical scavenging activity</th>
<th>Superoxide anion radical scavenging activity</th>
<th>Hydroxyl radical scavenging activity</th>
<th>15-Lipoxygenase inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-l</td>
<td>52.0 ± 0.5*</td>
<td>0.73 ± 0.00**</td>
<td>1.26 ± 0.05**</td>
<td>154.6 ± 0.6*</td>
</tr>
<tr>
<td>S-f</td>
<td>46.7 ± 0.2*</td>
<td>0.65 ± 0.01**</td>
<td>1.6 ± 0.0**</td>
<td>154.86 ± 0.35*</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>5.5 ± 0.0*</td>
<td>2.36 ± 0.03**</td>
<td>0.3 ± 0.0**</td>
<td>29.2 ± 0.2*</td>
</tr>
</tbody>
</table>

S-l – leave extract; S-f – flower extract
* results expressed in μg/mL; ** results expressed in mg/mL.

**DISCUSSION**

This *in vitro* screening compared the free radical scavenging and 15-lipoxygenase inhibitory effects of *Crataegus succulenta* extracts with those of (+)-catechin. Due to the o-dihydroxy moiety, catechin is one of the most potent free radical scavengers (10, 11). In addition, catechin has been reported to be an efficient inhibitor of 15-lipoxygenase (12). It is worthy to note that, in superoxide anion radical scavenging assay, both extracts were more active than (+)-catechin. In hydroxyl radical scavenging and 15-lipoxygenase inhibition assays, the extracts were only 4-5 times less active than (+)-catechin. In light of these data, it is obvious that *Crataegus succulenta* extracts are efficient free radical scavengers and 15-lipoxygenase inhibitors. These effects suggest potential therapeutic applications for *Crataegus succulenta* extracts. Reactive oxygen species such as superoxide anion and hydroxyl radicals are involved in the development of many chronic diseases (cardiovascular, neurodegenerative and malignant diseases) (5). 15-Lipoxygenase is a prooxidant enzyme; it catalyzes the oxidation of low-density lipoproteins (LDL) being a source of free radicals responsible for non-enzymatic oxidative processes. 15-Lipoxygenase is involved in the development of atherosclerosis, prostate cancer, diabetes, Alzheimer's and Parkinson's diseases (13).

This study revealed no striking differences in total phenolic, flavonoid and proanthocyanidin contents between *Crataegus succulenta* extracts. According to the EC$_{50}$ values, both extracts showed similar 15-lipoxygenase inhibitory activities but different free radical scavenging effects. S-f scavenged more effectively DPPH and superoxide radicals while S-l was more active against hydroxyl radical. A lack of concordance between the antioxidant effects of *Crataegus succulenta* extracts is noticeable. Such a lack of concordance between the antioxidant properties evaluated by different assays has already been mentioned in literature and might have two possible explanations: first, the different chemical profile of the antioxidant constituents in plant extracts that significantly affects the antioxidant activity and second, the complexity of the mechanisms involved in antioxidant activity (14).

**CONCLUSIONS**

The results of this study suggest the possibility that extracts from the leaves and
flowers of *Crataegus succulenta* could be used as ingredients in functional foods for the prevention of oxidative stress-related diseases. Further research is required to investigate the *in vivo* efficacy and safety of the extracts.

**ACKNOWLEDGEMENTS**

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**REFERENCES**