© 2018 Journal of Pharmacy & Pharmacognosy Research, 6 (2), 89-95, 2018
ISSN 0719-4250
http://jppres.com/jppres

Original Article | Artículo Original

Pinus sylvestris L. and other conifers as natural sources of ascorbic acid

[*Pinus sylvestris* L. y otras coníferas como fuentes naturales de ácido ascórbico]

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Abstract

Context: There is a widespread opinion that needles of conifers are a good source of ascorbic acid and may help to cure and prevent scurvy.

Aims: To determine the content of ascorbic acid in needles and shoots of *Pinus sylvestris*, *Picea abies*, *Juniperus communis* and several other conifers.

Methods: Ascorbic acid content was analyzed by HPLC: aminocolumn 250 mm x 4.6 mm, 5 µm; mobile phase acetonitrile and 0.05 M KH_2PO_4 (75:25), flow rate 1.0 mL/min, detection at 266 nm.

Results: The fresh needles contained more (10.8 x 10^{-3}%) of ascorbic acid than the fresh shoots (5.4 x 10^{-3}%) of the same *P. sylvestris* tree. In needles collected during one year with monthly intervals from the same pine tree the content of vitamin C varied from 0.5 x 10^{-3}% to 15.7 x 10^{-3}%. The needles gathered in winter from November to March contained much more of ascorbic acid (5.2-15.7 x 10^{-3}%) than needles collected during warmer season (0.5-2.8 x 10^{-3}%). The fresh needles of compared conifers (n=11) contained ascorbic acid from 0% (*Tuja occidentalis*) to 15.0 x 10^{-3}% (*Tsuga canadensis*), needles of *J. communis* contained ascorbic acid a little more (13.3 x 10^{-3}%) than needles of *P. sylvestris, P. abies* and *Microbiota decussata* (9.0-10.8 x 10^{-3}%).

Conclusions: The concentration of ascorbic acid in needles and shoots of studied conifers is rather low if compared to other natural sources of vitamin C. Fresh plant material of *P. sylvestris, P. abies* and *J. communis* contained more ascorbic acid than frozen material. The concentration of ascorbic acid in needles of these three trees was higher than in shoots.

Keywords: HPLC analysis; needles; Scots pine; scurvy; shoots; vitamin C.

Resumen

Contexto: Existe una opinión generalizada de que las agujas de coníferas son una buena fuente de ácido ascórbico y pueden ayudar a curar y prevenir el escorbuto.

Objetivos: Determinar el contenido de ácido ascórbico en agujas y brotes de *Pinus sylvestris*, *Picea abies*, *Juniperus communis* y varias otras coníferas.

Métodos: El contenido de ácido ascórbico se analizó por HPLC: columna amino de 250 mm x 4,6 mm, 5 µm; acetonitrilo en fase móvil y KH_2PO_4 0,05 M (75:25), caudal 1,0 mL/min, detección a 266 nm.

Resultados: Las agujas frescas contenían más ácido ascórbico (10,8 x 10^{-3}%) que los brotes frescos (5,4 x 10^{-3}%) del mismo árbol de *P. sylvestris*. En las agujas recolectadas durante un año con intervalos mensuales del mismo árbol de pino, el contenido de vitamina C varió de 0,5 x 10^{-3}% a 15,7 x 10^{-3}%. Las agujas recolectadas en el invierno de noviembre a marzo contenían mucho más ácido ascórbico (5,2-15,7 x 10^{-3}%) que las agujas recolectadas durante la temporada más cálida (0,5-2,8 x 10^{-3}%). Las agujas frescas de las coníferas comparadas (n = 11) contenían ácido ascórbico del 0% (*Tuja occidentalis*) a 15,0 x 10^{-3}% (*Tsuga canadensis*), las agujas de *J. communis* contenían más ácido ascórbico (13,3 x 10^{-3}%) que las agujas de *P. sylvestris*, *P. abies* y *Microbiota decussata* (9,0-10,8 x 10^{-3}%).

Conclusiones: La concentración de ácido ascórbico en las agujas y los brotes de las coníferas estudiadas es bastante baja si se compara con otras fuentes naturales de vitamina C. El material vegetal fresco de *P. sylvestris*, *P. abies* y *J. communis* contenía más ácido ascórbico que el material congelado. La concentración de ácido ascórbico en las agujas de estos tres árboles fue más alta que en los brotes.

Palabras Clave: agujas; análisis por HPLC; brotes; escorbuto; *Pino silvestre*; vitamina C.

ARTICLE INFO
Received: July 4, 2017.
Received in revised form: October 26, 2017.
Accepted: November 1, 2017.
Available Online: December 6, 2017.
Declaration of interests: The authors declare no conflict of interest.
Funding: The authors confirm that the project has no funding or grants.

INTRODUCTION

Humans are not able to synthesize vitamin C (ascorbic acid), and scurvy (scorbut) is an acute, chronic illness caused by a dietary deficiency of this vitamin, it repeatedly caused morbidity and death. Historically, during the second voyage (1535-1536) of Jacques Cartier the sickness began to spread in all three of his ships, and of 110 crew members, 8 were dead and about 50 past all hope of recovery. For the cure of scurvy, they used Iroquois decoction from bark and leaves of the "tree of life" known later as an "Annedda". They learned from Indians to cure the scurvy using pine needles and bark (Durzan, 2009; McDowell, 2013). Afterwards, the name of Annedda was narrowed down to different *Pinus* and *Picea* species as well as to *Juniperus communis, Tsuga canadensis, Thuja occidentalis*, among others. The successful cure for scurvy was very significant for future naval explorations (Durzan, 2009).

During Swedish-Russian War in the 18th Century, almost all Swedish soldiers were severely affected by scurvy, after drinking a solution prepared from pine needles their health was restored. During the 19th and 20th Centuries the young shoots of pine and spruce were used in Canada, Norway, and Russia against scurvy (McDowell, 2013).

It was estimated that during the Leningrad Blockade (1941-1944) about 1-1.5 million of people died. One of the causes of the death in addition to severe starvation was the scurvy induced by the deficiency of vitamin C as there was no fresh vegetarian food available. Therefore, the scientists worked to extract vitamin C from pine (*Pinus sylvestris* L.) needles to help to cure and prevent the outbreaks of scurvy (Salisbury, 1985; Pleysier, 2008). Full-scale collection of pine needles was started, and the anti-scurvy drink made from those was made available in every public catering establishment, school and factory canteen. Overall about 16 million doses of life-saving drink were consumed during the year 1942 (Moskoff, 2002). In his book, Collingham (2011) communicates the memories of a blockade survivor Vera Miljutina accordingly to what the drink cooked of pine needles had made miracles. In especially severe situations people even chewed those pine needles saving the time and trouble to boil the water. It is an interesting observation that people who had consumed for a long time larger quantities of ascorbic acid before the blockade had tendency to die sooner than those who had grown used to smaller quantities (Rhead and Schrauzer, 1971).

Also, nowadays it is a typical myth that the shoots of *P. sylvestris* and *P. abies* are rich of ascorbic acid and tea made of those could be used as a vitamin drink during military trainings in extreme situation in forest encampment (Haljand, 2001).

In earlier studies we investigated the content and composition of essential oil in shoots of *P. sylvestris* and *J. communis* growing in Estonia (Raal et al., 2010; 2015), this paper was focused on the level of ascorbic acid in conifers of Estonian origin. The aim of this study was to determine the content of ascorbic acid in the needles and shoots of *P. sylvestris* and other conifers.

MATERIAL AND METHODS

Plant material

The needles and shoots of Scots pine were collected from different places in Estonia. To study the correlation between the content of ascorbic acid and the location of growth of a particular pain tree, needles were collected form 11 different locations for one month (August/September 2014) (Table 1). To study the dynamics of the content of ascorbic acid in pine needles, the herbal material was collected on the same date during one year from a pine tree located in Tartu city (N 58° 21'66.7'', E 26° 41'31.8''). In addition to this, the content of ascorbic acid was assayed during two weeks in the needles and shoots of one Scots pine located in Soinaste, Ülenurme parish, county of Tartumaa (N 58° 20'78.1'', E 26° 41'69.4''). The needles and shoots of *Picea abies* and *Juniperus communis* were collected from three counties (Table 2). Other studied conifers were from the Botanical Garden of University of Tartu (N 58°23'05'', E 26°43'12''), which had been identified beforehand by the botanists of the Garden. The *Pinus sylvestris* L., *Picea abies* (L.) H.Karst. and *Juniperus communis* L. as very common trees in Estonia were identified using the taxonomic guide (Krall et al., 2007). The voucher specimens of *P. sylvestris* and *J. communis* were deposited at the Institute of Pharmacy, University of Tartu, Estonia

(No Pinaceae/Pin6 and Cypressaceae/Jun9, respectively). The samples of other conifers were not deposited at the Institute, these original trees/shrubs studied were labeled in the Botanical Garden of the University of Tartu. The content of ascorbic acid was assayed in fresh (immediately after gathering) or frozen (-18 °C) material.

Table 1. Content of ascorbic acid in *P. sylvestris* needles from different habitats.

Habitat	Coordinates	Content of ascorbic acid (x10^{-3}%)
Tartu, Tartu county	N 58°21'66.7"E 26°41'31.8"	0.5 ± 0.1^{a}
Kuusalu parish, Harju county	N 59°25'55.8"E 25°23'09.8"	1.3 ± 0.1^{a}
Rakvere parish, Lääne-Viru county	N 59°22'06.2"E 26°20'12.6"	1.3 ± 0.1^{a}
Viiratsi parish, Viljandi county	N 58°22'05.5"E 25°38'46.9"	1.9 ± 0.2
Kose parish, Harju county	N 59°05'50.9"E 25°22'40.8"	2.0 ± 0.2
Raikküla parish, Rapla county	N 58°57'04.0"E 24°40'13.1"	2.1 ± 0.2
Varbla parish, Pärnu county	N 58°21'40.1"E 23°44'42.5"	2.7 ± 0.3
Paikuse, Pärnu county	N 58°22'28.8"E 24°37'09.6"	3.4 ± 0.3^{b}
Torma parish, Jõgeva county	N 58°51'41.9"E 26°51'04.2"	3.6 ± 0.3^{b}
Kilingi-Nõmme, Pärnu county	N 58°09'36.9"E 24°59'00.8"	4.1 ± 0.4^{b}
Tõrva, Valga county	N 57°59'40.5"E 25° 56'14.1"	6.7 ± 0.6^{b}

Data represent mean ± SD of three samples in each group. Values with different letters in superscripts are statistically significant at p ≤ 0.01 using two-tailed t-Test for independent variables. ^{a} - less than median value 2.1 ± 0.2 x 10^{-3}% , ^{b} - more than 2.1 ± 0.2 x 10^{-3}%.

Table 2. Content of ascorbic acid in fresh and frozen needles and shoots of *Pinus sylvestris, Picea abies* and *Juniperus communis.*

Plant	Content of ascorbic acid (x10^{-3}%)		Collected from
	Needles	Shoots	
Fresh plant material			
Picea abies	9.00 ± 0.80^{a}	1.50 ± 0.10^{a}	Ülenurme, Tartu county (N 58° 19'13,0"E 26° 41'39,5")
Pinus sylvestris	10.80 ± 1.00	5.40 ± 0.50^{a}	Ülenurme, Tartu county (N 58° 20'78,1" E 26° 41'69,4")
Juniperus communis	21.70 ± 2.00	na	Tartu (N 58°23'05,7" E 26°43'12,3")
Frozen plant material			
Juniperus communis	-	na	Raikküla, Rapla county (N 58°54'14,0"E 24°40'58,3")
Picea abies	0.40 ± 0.04	0.10 ± 0.01^{a}	Raikküla, Rapla county (N 58°54'14,0"E 24°40'58,3")
Picea abies	1.40 ± 0.10	0.10 ± 0.01^{a}	Paikuse, Pärnu county (N 58°22'28,8"E 24°37'09,5")
Picea abies	2.50 ± 0.20	0.50 ± 0.01^{a}	Puka, Valga county (N 58°05'34,5"E 26°05'43,4)
Pinus sylvestris	4.10 ± 0.40	0.20 ± 0.02^{a}	Ülenurme, Tartu county (N 58° 20'78,1" E 26° 41'69,4")
Pinus sylvestris	4.20 ± 0.40	-	Ülenurme, Tartu county (N 58° 20'78,1" E 26° 41'69,4")

na – not analyzed. Data represent mean ± SD of three samples in each group. Values with ^{a} in superscript in the same row are statistically significant at the p≤0.01 level using two-tailed t-Test for independent variables.

Preparation of herbal material for analysis

The fresh herbal material was cut using scissors into pieces. To one part of herbal material 20 parts of 1% citric acid solution in water at room temperature was added, and the plant material was ground in a mortar for five minutes. The 1% citric acid solution also contained 3% (V/V) acetonitrile to inhibit the decomposition of ascorbic acid. After processing in a mortar, the samples were kept for 5 min at the ultrasonic bath, then placed into centrifuge tubes and centrifuged at 10 000 *g* for 10 minutes. After centrifugation, the samples were filtered through 25 mm diameter 0.45 µm acetate membrane filter and ~1 mL aliquots dispensed into HPLC autosampler 2 mL vials. The content of ascorbic acid was calculated in %(m/m) for absolutely dry drug. The loss on drying was determined by European Pharmacopoeia (2010) for fresh and frozen plant material. The data were presented as an averaged result of three parallel experiments with standard deviation.

Assay of ascorbic acid

The content of ascorbic acid was determined by a slightly modified European Pharmacopoeia HPLC method for quantification of related substances in ascorbic acid as API (European Pharmacopoeia 2010: Monograph 01/2011:0253). The details of the method were published in our earlier paper (Meos et al., 2017). Aminocolumn 250 mm x 4.6 mm, stationary phase particle size 5µm (Phenomenex Luna® NH2) was used. The mobile phase was a mixture of acetonitrile and 0.05M aqueous solution of potassium-dihydrogen-phosphate 75:25 (V/V), flow rate 1.0 mL/min, column temperature 45°C, detection wavelength 266 nm, 10 µL of sample was injected. The assay was performed on HPLC Shimadzu LC 20 Prominence, with photo-diode array detector SPD-M20A and Nexera X2 SIL-30AC autosampler. The system was controlled, data collected and processed by LabSolutions 5.71 SP1 software. Commercial reagent L-ascorbic acid (Sigma, 255564) was used as an ascorbic acid reference substance. Immediately before the assay, ~0.2 mg/mL reference solution was made with 5% (V/V) acetonitrile aqueous solution.

To test the linearity between the concentration of standard and the area of the corresponding peak a calibration curve was built. The correlation coefficient between these parameters within the ascorbic acid concentration range from 0.50 to 0.01 mg/mL was close to 1 ($y = 0.00000x + 0.00103$, $R^2 = 0.99998$, $r = 0.99999$).

Statistical analysis

All the data were presented as a mean of three parallel analyses with its standard deviation. For statistical analysis of the results basic parametric statistics of two-tailed t-Test for independent variables assuming unequal variances was used.

RESULTS

Ascorbic acid in *P. sylvestris* needles from different habitats

The content of ascorbic acid in the *P. sylvestris* needles collected from 11 different habitats during a month in September and October was from 0.5×10^{-3}% to 6.7×10^{-3}%, analyzed in frozen material and calculated on dry plant material (Table 1). Difference between maximum and minimum content of ascorbic acid was more than 13-fold. The largest concentration of ascorbic acid ($6.7 \pm 0.6 \times 10^{-3}$%) was found in pine needles collected from Tõrva, the rest of the samples contained ascorbic acid mostly between 2 and 4×10^{-3}%.

Dynamics of ascorbic acid in *P. sylvestris* needles during a year

In needles collected during one year with monthly intervals from the same pine tree, the content of vitamin C varied from 0.5×10^{-3}% to 15.7×10^{-3}%, i.e. more than 30 times (Table 3). The needles gathered in winter from November to March contained much more ($p<0.009$) of ascorbic acid (5.2-15.7 $\times 10^{-3}$%) than needles collected during warmer season (0.5-2.8 $\times 10^{-3}$%).The highest concentration of ascorbic acid was found in the needles in February and March (13.3×10^{-3}% and 15.7×10^{-3}%, respectively). In May, July, August, September, and October the content of ascorbic acid was less than 1.5×10^{-3}%. The ascorbic acid content was the smallest in September and May, 0.5 ± 0.05 and $0.7 \pm 0.07 \times 10^{-3}$% accordingly. It has been found that plants grown geographically in the North as well as cold-short plants accumulated more ascorbic acid. It is possible that vitamin C (probably in the form of dehydroascorbic acid) could be one of the factors that protect plant cells form adverse effects of cold (Troshin, 1967).

Table 3. Dynamics of ascorbic acid content in *P. sylvestris* needles during a year.

Month of collection	Content of ascorbic acid ($x10^{-3}$%)
January*	8.1 ± 0.8[a]
February	13.3 ± 1.3[a]
March	15.7 ± 1.5[a]
April	2.2 ± 0.2
May	0.7 ± 0.1
June	2.8 ± 0.3
July	1.4 ± 0.1
August	1.2 ± 0.1
September	0.5 ± 0.1
October	1.0 ± 0.1
November	5.2 ± 0.5[a]
December	8.7 ± 0.8[a]

*Always the day of the month was the same (16th). Data represent mean ± SD of three samples in each group. [a] superscript shows the mean difference of pooled data from November to March is statistically significant at $p \leq 0.009$ level compared to pooled data from April to October using two-tailed t-Test for independent variables.

Ascorbic acid in needles and shoots of *Pinus sylvestris*, *Picea abies* and *Juniperus communis*

According to State Pharmacopoeia of U.S.S.R. (1989), the shoots of *P. sylvestris* were the officinal crude drug in the pharmacy of Soviet Union. By the monograph of *Gemmae Pini,* the plant material should contain not less than 0.3% of essential oil. Regarding *P. abies* and *J. communis* only the fruits of juniper (*Fructus juniperi*) are in use (State Pharmacopoeia of U.S.S.R., 1989). Traditionally Russians used fermented *P. sylvestris* shoots against scurvy (Spary, 2000). In this study, the fresh needles of *J. communis, P. sylvestris* and *Picea abies* contained 21.7 x 10^{-3}%, 10.8 x 10^{-3}% and 9.0 x 10^{-3}% of ascorbic acid accordingly.

There was a tendency that the fresh needles contained more (10.8 x 10^{-3}%, $p<0.056$) of ascorbic acid than the fresh shoots (5.4 x 10^{-3}%) of the same *P. sylvestris* tree (Table 2). Similarly, the needles of *P. abies* contained ascorbic acid more ($p<0.018$) than the shoots of the same tree. Our earlier preliminary study showed that frozen pine, spruce, and juniper needles contained ascorbic acid accordingly 3.4 ± 0.3 x 10^{-3}%, 1.9 ± 0.1 x 10^{-3}% and 2.2 ± 0.2 x 10^{-3}%, at the same time frozen shoots of these conifers contained it between zero and 0.5 x 10^{-3}%.

Ascorbic acid in fresh needles of different conifers

Comparative data of the ascorbic acid content in fresh needles of different conifers is presented in Table 4. There was no vitamin C in *Thuja occidentalis* as well as the content was very low in *Thujopsis dolabrata* (0.1 ± 0.01 x 10^{-3}%). *Abies concolor, Larix decidua,* and *Chamaecyparis pisifera* contained usual amount of ascorbic acid (1.1-4.4 x 10^{-3}%). The largest content of ascorbic acid (15.0 ± 1.4 x 10^{-3}%) was found in *Tsuga canadensis.* The needles of *Picea abies* contained ascorbic acid practically on the same level with *Taxus baccata, Microbiota decussate* and *Pinus sylvestris.* Hence the content of ascorbic acid in the needles of *P. sylvestris* was not quite high compared to other conifers studied by us.

Table 4. Content of ascorbic acid in fresh needles of different conifers.

Conifer	Ascorbic acid ($x10^{-3}$%)
Thuja occidentalis L.*	-
Thujopsis dolabrata (L.f.) Siebold et Zucc*	0.10 ± 0.01[a]
Abies concolor (Gordon) Lindl. ex Hildebr.*	1.10 ± 0.10[a]
Larix decidua Mill*	3.90 ± 0.40[a]
Chamaecyparis pisifera (Siebold et Zucc.) Endl.*	4.30 ± 0.40[a]
Taxus baccata L.*	8.60 ± 0.80[b]
Picea abies (L.) H.Karst.	9.00 ± 0.80
Microbiota decussata Kom.*	9.60 ± 0.90
Pinus sylvestris L.	10.80 ± 1.00
Juniperus communis L.*	13.30 ± 1.30
Tsuga canadensis (L.) Carrière*	15.00 ± 1.40[c]

* Collected from the Botanical Garden of the University of Tartu. Data represent mean ± SD of three samples in each group. Values with different letters in superscript are statistically significant at [a] $p \leq 0.01$ or [b,c] $p \leq 0.05$ using two-tailed t-Test for independent variables. [a,b] - less than value for *Pinus sylvestris* L. 10.80 ± 1.00 x 10^{-3}% , [c] - more than 10.80 ± 1.00 x 10^{-3}%.

DISCUSSION

The content of ascorbic acid in the needles and shoots of *P. sylvestris, P. abies,* and *J. communis* was quite low in this study. For example, the variability of ascorbic acid content in the needles of *P. sylvestris* from different geographical regions of Middle Siberia, Soviet Union, was 171–217 x 10^{-3}% (*versus* 15.7 x 10^{-3}% or less in fresh needles in this study), although these results were obtained using titration with 2,6-dichlorophenolindophenol (Zubareva et al., 2011). The ascorbic acid content in green spruce needles from East Bavarian mountains, Germany, was between 1.3 and 2.7 mg/g (130–270 x 10^{-3}%) dry weight (Osswald et al., 1987).

For comparison, in fresh leaves of *Primula veris* collected with weekly interval the content of ascorbic acid varied from 1.19 to 2.39%, being highest from mid of May to mid of June. It is interesting to notice that the ascorbic acid level in fresh plant material was several times higher than in frozen needles or shoots of same trees. Our earlier study showed that in case of common cowslip the content of ascorbic acid did not decrease even when stored for one year at a temperature of -18°C (Meos et al., 2017).

At the same time, the present experiments showed rather low level of ascorbic acid in different conifers studied. According to Rousseau (1954), the fresh needles of *Thuja occidentalis* contained 45 x 10^{-3}%, *Abies balsamea* 270 x 10^{-3}%, *Picea rubens* 169 x 10^{-3}% and *Pinus strobus* 32 x 10^{-3}% of ascorbic acid determined titrimetrically. So, the difference between the results of two different analytical methods could be more than two magnitudes.

According to this study, the average content of ascorbic acid in dried needles of pine collected from different habitats was 2.7 x 10^{-3}%, and up to 15.7 x 10^{-3}% in fresh needles. To maintain the recommended daily intake (60 mg), one should consume every day 2.222 kg dried pine needles, which is not quite realistic. In fact, even that could not be enough, as the adult scurvy is nowadays treated with 300-1000 mg of ascorbic acid per day (McPhee and Papadakis 2007). Ascorbic acid is recommended in clinical dermatology three times a day, 100 mg until 4 g is reached, and after that 100 mg per day in days to weeks (Ficzpatrick et al., 2001). A medium-size apple has been estimated to contain 6-8 mg of vitamin C. The mean daily intake of ascorbic acid from potatoes was at least 17 mg in a working-class household in Great Britain during 1909-1949 (Carpenter, 1988).

At the same time, the human dietary studies of experimentally induced scurvy conducted during WW II in Britain and on Iowa state prisoner "volunteers" in the late 1960s suggest that much less than recommended a daily dose of ascorbic acid could cure this disease. All obvious symptoms of scurvy could be completely reversed by additional vitamin C supplementation of only 10 mg per day. No clinical difference was noted between men given 70 mg vitamin C per day, and those given 10 mg per day (Pemberton, 2006). Men on a diet devoid or nearly devoid of vitamin C had signs of scurvy and were estimated to have a body pool of less than 300 mg, with a daily turnover of only 2.5 mg/day (Hodges et al., 1969). Thus, in case of Leningrad Blockade when fresh *P. sylvestris* were collected in winter time, the concentration of ascorbic acid in those seems to be enough to cure scurvy.

CONCLUSIONS

The concentration of ascorbic acid in needles and shoots of *Pinus sylvestris* and other conifers is rather low if compared to other natural sources of vitamin C. Fresh plant material of *P. sylvestris, Picea abies* and *Juniperus communis* contained more ascorbic acid than frozen material. The concentration of ascorbic acid in needles of those three conifers was higher than in shoots. The *P. sylvestris* needles gathered in winter contained much more of ascorbic acid than needles collected during the warmer season.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

The authors confirm that the project has no funding or grants.

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Author contribution:

Contribution	Raal A	Nisuma K	Meos A
Concepts or ideas	X		X
Design	X		
Definition of intellectual content	X	X	X
Literature search	X	X	X
Experimental studies	X	X	X
Data acquisition	X	X	X
Data analysis	X	X	X
Statistical analysis			X
Manuscript preparation	X		X
Manuscript editing	X		X
Manuscript review	X	X	X

Citation Format: Raal A, Nisuma K, Meos A (2018) *Pinus sylvestris* L. and other conifers as natural sources of ascorbic acid. J Pharm Pharmacogn Res 6(2): 89–95.