

EXPERIMENTAL PAPER

Estimation of total phenolics and flavonoidal contents as well as *in vitro* antioxidant potential of *Apium leptophyllum* Pers.

HIMANSHU BHUSAN SAHOO¹, SUBRAT KUMAR BHATTAMISRA², UTTAM KUMAR BISWAS¹, RAKESH SAGAR^{3*}

¹Department of Pharmaceutical Sciences
NIMS University
Jaipur, 303121, Rajasthan, India

²Roland Institute of Pharmaceutical Sciences
Berchampur
760010 Odisha, India

³Vedica College of Pharmacy
Bhopal 462033, MP, India

*corresponding author: e-mail: drrsagar@gmail.com, phone: +91 9098999173

Summary

At present, major causes of diseases is oxidative stress affecting both metabolic and physiological functions of the body. That is why there is a great need for investigation of nutritious food supplements for counteracting these oxidative stresses. Therefore, the aim of study was to evaluate the therapeutic potential of *Apium leptophyllum* Pers. fruits by estimating total phenolic as well as flavonoidal contents and antioxidant values. The collected fruits were extracted separately using different solvents like methanol, ethanol and water. Total phenolic and flavonoid contents were measured from the respective extracts and correlated with their antioxidant values. The antioxidant properties of various fruit extracts (12.5, 25, 50, 100 and 150 $\mu\text{g/ml}$) were evaluated by DPPH, hydroxyl, nitric oxide and superoxide scavenging assay and compared with ascorbic acid as a standard. All the extracts of *A. leptophyllum* were found to be dose dependent inhibition against these free radicals. Among all these extracts, the methanolic one was found better in the scavenging activity and followed dose-dependent manner against DPPH, hydroxyl radical, nitric oxide, superoxide anions with minimum IC_{50} values of 97.9, 89.02, 135.37, 127.73 $\mu\text{g/ml}$, respectively, and also observed more significant ($p < 0.01$) as compared with standard.

Furthermore, total phenolic and flavonoidal contents were found highest in methanolic extract. The results obtained in this study clearly indicate that the methanolic extract of *A. leptophyllum* may be used as a new potential source of natural nutritional supplement in food or pharmaceutical industries due to rich source of phenolic, flavonoidal contents as well as antioxidant property.

Key words: *Apium leptophyllum*, antioxidant value, IC_{50} value, total phenolic and flavonoid contents

INTRODUCTION

In world population, the majority of diseases is directly or indirectly associated with oxidative stress caused by free radicals in biological systems [1]. These free radicals cause oxidation of biomolecules like proteins, amino acids, lipids, DNA in living organisms; leads to cell injury, aging of organisms, DNA damage, arthritis, cancer promotion, cardiovascular diseases and other neurodegenerative diseases [2-5]. These radicals are produced by ultraviolet light, ionizing radiation, chemical reactions and metabolic processes in the living system and implying its harmful effects on living organisms [6]. The antioxidants are vital substances, which possess ability to protect the body from harmful damage by inhibiting lipid peroxidation or interfering oxidation process with free radicals or catalytic metals [7-9].

Apium leptophyllum Pers. Mull. belongs to *Umbelliferae* family, popularly known as Ajmuda [10]. It is generally distributed throughout India, Sri Lanka, Pakistan, South America, Queensland, tropics etc. In India, it is an annual herb up to 50 cm height, cultivated in Andhra Pradesh, Gujarat, Madhya Pradesh, Jammu-Kashmir, Karnataka etc. [11, 12]. In the traditional system of Ayurvedic medicine, the fruit part of this plant is widely used as antinephritic, antirheumatic, carminatives and also useful in the prevention of tumor, anorexia, vomiting and colic pain [13-17]. In Ethiopian traditional medicines, the leaves of *Apium leptophyllum* were used for the treatment of a disease locally known as mitch, which is characterized mainly by inflammation, sweat and loss of appetite. The volatile oil from the leaves possesses antimicrobial activity against a broad spectrum of pathogens including gram-positive and gram-negative bacteria as well as some fungal strains and also observed excellent *in vitro* radical scavenging activity against DPPH only [18, 19]. Phytochemically, it contains volatile oils, coumarin derivatives, terpene hydrocarbons, phenolics, alkaloids etc [20-22]. However, there is no scientific report available on antioxidant activity from fruit parts of *Apium leptophyllum*. Therefore, present investigation was undertaken to examine the antioxidative potential of various fruit extracts through various *in-vitro* radical scavenging models and correlated with their total phenolic and flavonoidal contents.

MATERIALS AND METHODS

Chemicals and reagents

2, 2-diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), gallic acid, catechin, nicotinamide adenine dinucleotide (NADH), phenazine meta sulphate (PMS), trichloroacetic acid (TCA) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich, Germany. Sodium carbonate, sodium nitroprusside solution and tri-chloro acetic acid (TCA) were purchased from SD Fine Chemicals, Mumbai. All other reagents used were of analytical grade.

Plant material

The fruits of *Apium leptophyllum* were collected from Bhopal District, Madhya Pradesh, India. Further taxonomic identification was conducted by Dr. Madhu Rajput, Herbarium in charge, Department of Botany, Jiwaji University, MP, India. A voucher specimen was deposited in the herbarium (F/HERB/2010/3405) for further investigation.

Extraction of plant material

The collected fruits of *A. leptophyllum* were cleaned, washed with water and reduced to coarse powder with a blender. For aqueous extraction, accurately weighed 10 gm of fresh coarse powdered sample was extracted with 100 ml boiling water into 500 ml of beaker by maceration for 48 hours. For methanolic extraction, 10 g of powdered samples was extracted with petroleum ether for defatting. After complete extraction, drug was removed, dried and filled into the soxhlet assembly for successive extraction with 150 ml of methanol for 48 hours to obtain methanolic extract. Similarly, another 10 g of powdered sample were extracted overnight with 150 ml of ethanol in a shaker at room temperature for 48 hours to obtain ethanolic extract. Then the respective extracts was filtered through Whatman No.1 paper, concentrated and dried under vacuum. The extracted samples filled in plastic bottle and stored at -20°C until used. The percentage yield of methanol, aqueous and ethanol extract was found to be 16.4% w/w, 18.8% w/w and 12.6 % w/w, respectively.

Estimation of total phenolic and flavonoid content

Total phenolic contents in each extract were estimated by Folin-Ciocalteu reagent. About 1 ml of each extract solution containing 1g of extract in

a volumetric flask was diluted with 46 ml of distilled water. About 1 ml of Folin-Ciocalteu reagent was added and mixed properly. After three minutes, 3 ml of 2% sodium carbonate was added to the mixture and allowed to stand for 3 h with intermittent shaking. The blue color was developed and the absorbance was measured at 760 nm. The concentration of total phenolic contents in each extract was expressed as milligram of gallic acid equivalent (GAE) per 100 gram of fresh weight [23].

The total flavonoid content in each extract was determined by the aluminum chloride colorimetric assay. An aliquot (1 ml) of each extract or standard solution of catechin was added to 10 ml volumetric flask with distilled water. Then 0.3 ml of 5 % NaNO_2 and 10 % aluminum trichloride was added to the flask. After 10 minute of mixing, 2 ml of 1 M NaOH was added and the volume make up to 10 ml with distil water. The prepared solution was mixed well and measured the absorbance against blank sample at 510 nm. The amount of total flavonoids content in each extracts was expressed as milligram of catechin equivalents (CE) per 100 gm of fresh mass [23].

Determination of DPPH radical scavenging activity

For measuring the scavenging activity of DPPH free radicals, about 0.1 mM solution of DPPH in ethanol was prepared and 1ml of this solution was added to 3 ml of each extract at different concentration range (12.5-150 $\mu\text{g/ml}$). After 30 minutes, the absorbance was measured at 517 nm and compared with standard ascorbic acid [24]. The DPPH radical scavenging activity was measured and calculated using the following equation:

$$\% \text{ radical scavenging activity} = [(A_0 - A_t) / A_0] \times 100,$$

where A_0 was the absorbance of the control (without extract) and A_t was the absorbance in the presence of the tested samples and standard.

Determination of hydroxyl radical scavenging activity

The stock solutions of EDTA (1 mM), FeCl_3 (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml EDTA, 0.01 ml of FeCl_3 , 0.1 ml of H_2O_2 , 0.36 ml of deoxyribose, 1 ml of each extract of different concentration (12.5-150 $\mu\text{g/ml}$) was mixed with 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was incubated at 37° C for 1 h, about 1 ml portion of each incubated mixture was taken and mixed with 1 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025 M NaOH BHA) to develop the pink chromogen. After colour development, the absorbance of each sample was measured at 532 nm using UV Spectrophotometer and compared with standard ascorbic acid [25, 26]. The hydroxyl radical scavenging activity of each

extract was determined as percentage of inhibition of deoxyribose degradation and was calculated using the following equation:

$$\% \text{ of inhibition} = [(A_0 - A_t)/A_0] \times 100,$$

where A_0 was the absorbance of the control (blank) and A_t was the absorbance in the presence of the sample of the each extract.

Determination of nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside (5 mM) in Phosphate buffer saline (PBS) was prepared and mixed with 3 ml of different concentrations (12.5-150 $\mu\text{g/ml}$) of each extract and incubated at 25°C for 2 h. After incubation, 1 ml of Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride) was added to each sample and the absorbance was measured at 546 nm using UV spectrophotometer and compared with standard ascorbic acid [27]. The percentage of inhibition was measured using following formula:

$$\% \text{ inhibition} = [(A_0 - A_t)/A_0] \times 100,$$

where A_0 was the absorbance of the control (blank) and A_t was the absorbance in the presence of each extract.

Determination of superoxide radical scavenging activity

The method is based on generation of superoxide radical (O_2^-) by auto-oxidation of hydroxylamine hydrochloride in the presence of NBT, which gets reduced to nitrite. Each extract (12.5–150 $\mu\text{g/ml}$) were taken separately in test tube. The reaction mixture consisting of 1 ml of (50 mM) sodium carbonate, 0.4 ml of (24 mM) NBT, 0.2 ml of 0.1 mM EDTA and 0.4 ml of (1 mM) of hydroxylamine hydrochloride solutions were added to each test tube. Then reaction mixture was allowed to incubate at 25°C for 15 min and reduction of NBT was measured at 560 nm and compared with ascorbic acid as a reference compound [28]. The percentage of inhibition was calculated according to the following equation:

$$\% \text{ of inhibition} = [(A_0 - A_t)/A_0] \times 100,$$

where A_0 was the absorbance of the control (blank) and A_t was the absorbance in the presence of each extract.

Calculation of IC_{50} and statistical analysis

The sample concentration providing 50% inhibition (IC_{50}) was calculated from the linear program of inhibition percentage against sample concentration, and it is the

sample concentration that has 50% inhibition. The values were expressed in triplicate as Mean \pm SD. Statistical data were determined by one way ANOVA and Duncan's multiple range test using software systat 7.0, to find out the level of significance.

RESULTS

Estimation of total phenolic and flavonoidal contents

Total phenolic content in methanol, aqueous and ethanol extract of *Apium leptophyllum* was found to be 69.1 ± 0.39 mg, 58.8 ± 0.29 mg and 49.3 ± 0.56 mg of gallic acid equivalents/100 g, respectively, whereas the total flavonoid content of methanol, aqueous and ethanol extract was found to be 32.4 ± 0.42 mg, 19.5 ± 0.31 mg and 29.8 ± 0.87 mg catechin equivalents/100 g, respectively as shown in figure 1. The results suggest that the methanolic extract had higher level of phenolic and flavonoidal contents as compared to other extracts.

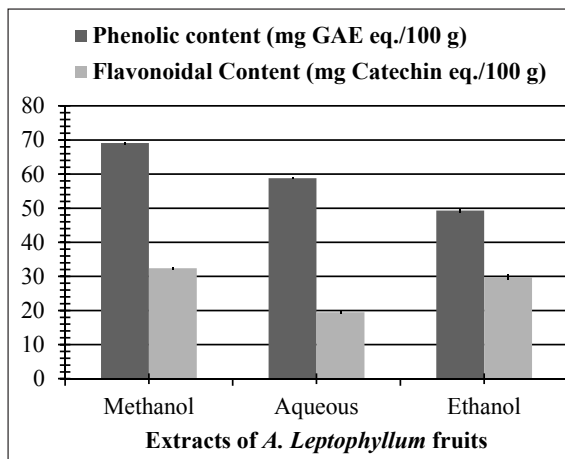


Figure 1.

Total contents of phenolics and flavonoids from various extracts of *A. leptophyllum* fruits

DPPH radical scavenging activity

The percentage of scavenging effect of DPPH on different extracts of *Apium leptophyllum* with a concentration of 12.5, 25, 50, 100 and 150 μ g/ml, was compared with ascorbic acid (fig. 2) and found dose dependent inhibitory antioxidant potential. Positive DPPH test suggests that the samples were free radical

scavengers. The IC_{50} values of methanolic, aqueous, ethanol extract and ascorbic acid were found to be 97.9, 123.52, 217.32 and 28.98 $\mu\text{g/ml}$, respectively (fig. 6). Among all these extracts, the methanol extract showed higher DPPH radical scavenging activity due to low IC_{50} value and the IC_{50} value was found significant ($p < 0.01$) scavenging effect as compared with ascorbic acid.

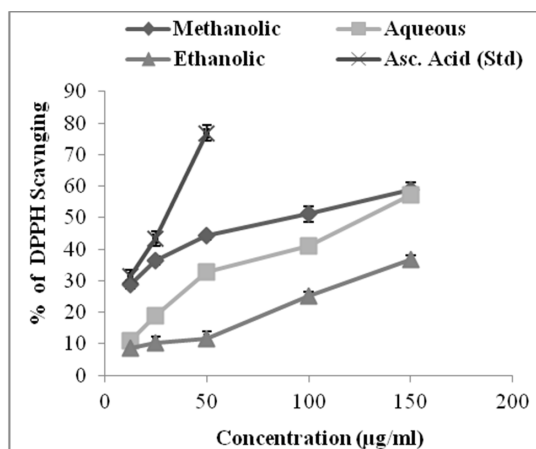


Figure 2.

DPPH scavenging activity of various extracts of *A. leptophyllum* fruits and ascorbic acid. All values were expressed in triplicate as mean \pm SD

Hydroxyl radical scavenging

The hydroxyl radical scavenging activity revealed that all extracts of *A. leptophyllum* from concentration range (12.5–150 $\mu\text{g/ml}$) showed the percentage of inhibition on hydroxyl radical scavenging activity in dose dependent manner (fig. 3). The ability of the abovementioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be a good scavenger of active oxygen species, thus reducing the rate of the chain reaction. The IC_{50} values of methanolic, aqueous, ethanol extract and ascorbic acid were found to be 89.02, 111.02, 195.22 and 84.25 $\mu\text{g/ml}$ respectively (fig. 6). The IC_{50} value of the methanolic extract was found very close with reference standard. Due to better hydroxyl radical scavenging ability, the methanolic extract showed very high antioxidant power.

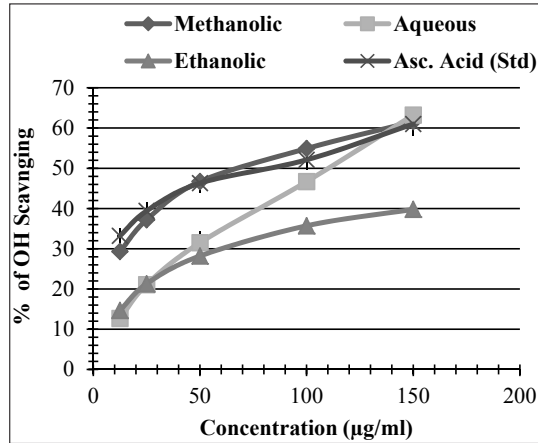


Figure 3.

Hydroxyl radical scavenging activity of various extracts of *A. leptophyllum* fruits and ascorbic acid. All values were expressed in triplicate as mean ± SD

Nitric oxide radical scavenging

In this study, the different extracts of *A. leptophyllum* were checked for inhibitory effect on nitric oxide production and showed good result in the inhibition of nitric oxide radical generation in a dose-dependent manner (fig. 4). The IC₅₀ values of methanolic, aqueous, ethanolic extract and ascorbic acid were found to be 135.37, 179.82, 282.81 and 73.22 µg/ml respectively (fig. 6). The IC₅₀ value of the all these extracts were found to be statistically significant ($p < 0.01$) with ascorbic acid.

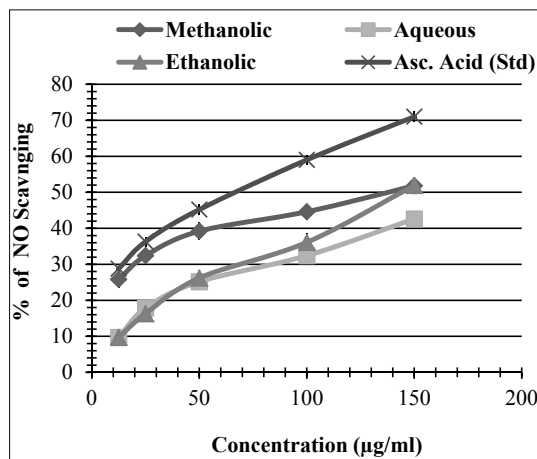


Figure 4.

Nitric oxide radical scavenging activity of various extracts of *A. leptophyllum* fruits and ascorbic acid. All values were expressed in triplicate as mean ± SD

Superoxide radical scavenging activity

In this study, different concentrations of each extract had followed strong superoxide scavenging activity in dose dependent manner (fig. 5). The IC_{50} values of methanolic, aqueous, ethanolic extract of *A. leptophyllum* and ascorbic acid were found to be 127.73, 144.01, 207.67 and 43.72 $\mu\text{g/ml}$, respectively (fig. 6). This result indicated that methanolic extract had strongly significant ($p < 0.01$) power than other extracts of *A. leptophyllum*.

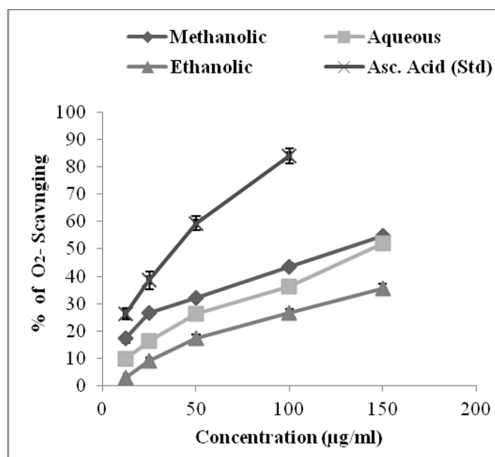


Figure 5.

Superoxide (O_2^-) radical scavenging activity of various extracts of *A. leptophyllum* fruits and ascorbic acid. All values were expressed in triplicate as mean \pm SD

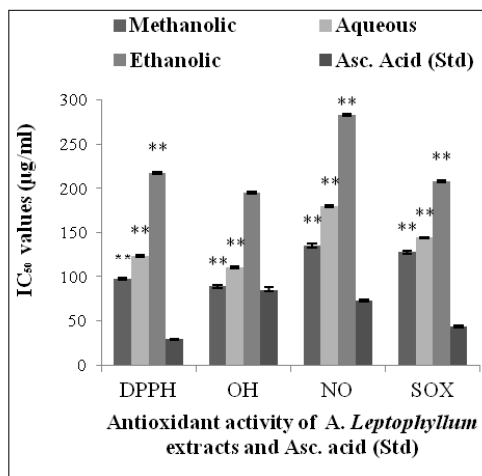


Figure 6.

Estimation of IC_{50} of various extracts of *A. leptophyllum* fruits and ascorbic acid (Std) by DPPH, OH, NO and SOX scavenging assay. All values were expressed in triplicate as mean \pm SD.

** $p < 0.01$ compared with std using one way ANOVA and Duncan's multiple range test.

DISCUSSION

Free radicals contain one or more unpaired electrons which are formed inside the biological environment. They are highly reactive and potential of the damage of biomolecules. These radicals are continuously produced in the human body because these are essential for detoxification, chemical signaling, supplying energy and immune functions. These are regulated by endogenous antioxidant enzyme system, but due to over production of these radicals leads to the risk to many diseases such as Alzheimer's disease, mild congestive impairment, Parkinson's disease, cardiovascular disorder, liver diseases, ulcerative colitis, inflammation, cancer etc [29]. So, there need strongly step towards reduction the risk of these chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defence or supplementing with proven antioxidants [30].

The phenolic compounds in herbs act as antioxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators [31]. Researchers have studied polyphenolic constituents having a potential to medicinal or nutraceutical properties including antioxidant activities [32]. Therefore, the study of the importance and role of non-nutrient compounds, particularly phenolic acids, flavonoids and high molecular tannins as natural antioxidants have greatly increased [33]. There was a linear relationship between the antioxidant activities and phenolic compounds [34-36]. The phenolic compounds are important constituents due to its scavenging power of hydroxyl groups [37, 38] and also improve the quality and nutritional value of food by retarding oxidative degradation of lipids [39-41]. Flavonoids and phenolic compounds were present in medicinal and dietary plants, which have been reported to exert multiple biological effects, including free radical scavenging abilities, anti-carcinogenic, antimutagenic, inhibition of hydrolytic and oxidative enzymes, blood glucose decreasing, anti-inflammatory activity etc. [42-47]. In our study it was revealed that the fruit extracts of *A. leptophyllum* are very promising sources of phenolic contents i.e. 49.3-69.1 mg of gallic acid equivalent and flavonoid contents 19.5-32.4 mg of rutin equivalent characterized by extremely high antioxidant activity.

The overproduction of free radicals such as DPPH, OH or NO leads to various types of chronic diseases afflicting to humans [48-50]. The hydroxyl radical has the capacity to join nucleotides in DNA and cause strand breakage which contributes to carcinogenesis, mutagenesis and cytotoxicity. It may cause oxidative damage to DNA, lipids and proteins [51-54]. Excess generation of nitric oxide is responsible for altering the structure and functional behavior of many cellular components by causing inflammation, cancer and other pathological conditions in human body. The extracts of *A. leptophyllum* showed antioxidant activity in competing with this free radical generation and found significant IC_{50} value as compared with standard.

CONCLUSIONS

From our investigation it can be concluded that the fruit extracts of *A. leptophyllum* showed strong antioxidant activity by inhibiting DPPH, hydroxyl, nitric oxide, superoxide radical scavenging activities when compared with standard ascorbic acid. However, the methanol extract of *A. leptophyllum* showed strong antioxidant activity having low IC_{50} value and high phenolic as well as flavonoidal content as compared to other extracts. Further studies in our laboratory are in progress for the isolation and identification of phytochemical compounds from methanol extract and to ensure that the medicinal value of the plant correlates with its antioxidant activity.

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OKREŚLENIE CAŁKOWITEJ ZAWARTOŚCI FENOLI I FLAWONOIDÓW ORAZ POTENCJAŁU ANTYOKSYDACYJNEGO *APIUM LEPTOPHYLLUM* PERS.

HIMANSHU BHUSAN SAHOO¹, SUBRAT KUMAR BHATTAMISRA², UTTAM KUMAR
BISWAS¹, RAKESH SAGAR^{3*}

¹Department of Pharmaceutical Sciences
NIMS University
Jaipur, 303121, Rajasthan, India

²Roland Institute of Pharmaceutical Sciences
Berchampur
760010 Odisha, India

³Vedica College of Pharmacy
Bhopal 462033, MP, India

*autor, do którego należy kierować korespondencję: e-mail: drrsagar@gmail.com,
tel.: +91 9098999173

Streszczenie

Obecnie główną przyczyną chorób jest stres oksydacyjny wpływający zarówno na metabolizm, jak i funkcje fizjologiczne organizmu. Dlatego istnieje duża potrzeba poszukiwania produktów uzupełniających dietę, które przeciwdziałają stresowi oksydacyjnemu. Celem niniejszej pracy było określenie potencjału terapeutycznego owoców *Apium leptophyllum* Pers. poprzez określenie całkowitej zawartości fenoli i flawonoidów oraz ich aktywności antyoksydacyjnej. Nasiona ekstrahowano oddzielnie, używając różnych rozpuszczalników, takich jak metanol, etanol i woda. Zmierzono całkowitą zawartość fenoli i flawonoidów w poszczególnych wyciągach i skorelowano ją z ich aktywnością antyoksydacyjną. Właściwości antyoksydacyjne różnych wyciągów z owoców (w stężeniach 12,5, 25, 50, 100 i 150 $\mu\text{g/ml}$) zmierzono za pomocą metod przy użyciu DPPH, rodników hydroksylowych, tlenku azotu, nadtlenu i porównano z właściwościami kwasu askorbowego. Stwierdzono, że działanie inhibicyjne wszystkich wyciągów z *A. leptophyllum* przeciwko wolnym rodnikom zależało od zastosowanej dawki. Ponadto całkowita zawartość związków fenolowych i flawonoidów była najwyższa w wyciągu metanolowym. Wyniki tych badań wyraźnie wskazują, że wyciąg metanolowy z *A. leptophyllum* może być używany jako potencjalne nowe źródło naturalnych suplementów diety dla przemysłu spożywczego lub farmaceutycznego dzięki wysokiej zawartości fenoli, flawonoidów i właściwościom antyoksydacyjnym.

Słowa kluczowe: *Apium leptophyllum*, wartość antyoksydacyjna, wartość IC_{50} , całkowita zawartość fenoli i flawonoidów