Int. J. New. Chem., 2021, Vol. 8, Issue 3, pp 265-276.

International Journal of New Chemistry

Published online 2021 in http://www.ijnc.ir/.

Open Access

Print ISSN: 2645-7236

Online ISSN: 2383-188x

Original Research Article

Isolation, Purification and Identification of β-carotene from *Azolla Pinnata* R. Br. as a New Carotenoid Wealthy Source

Sajjad Ghahari, Somayeh Ghahari, and Ghorban Ali Nematzadeh*

Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University, Iran

Received: 2020-02-23 *Accepted: 2020-06-21* *Published: 2021-07-01*

ABSTRACT

Azolla is a wealthy source of β-carotene. The purpose of this study was to isolate, purify and identify the β-carotene in *Azolla Pinnata* R. Br. plant. In this order, dry and fresh of *A. Pinnata* R. Br. were used and their β-carotene was extracted using the Acetone/Hexane solvent system. For the purification of β-carotene extracted, the column chromatography was used. In the following, an HPLC technique optimized for β-carotene designation and compared with a spectrophotometric standard method. The most appropriate sample conditions were: extraction with Hexane/Acetone 60:40 (v/v) and MeOH/THF/Water 67:27:6 (v/v) as mobile phase. The results indicated that the fresh sample has almost two times higher β-carotene comparing to the dried sample. So due to the importance of β-carotene as an antioxidant in one hand and the free of cost of mass production of *A. Pinnata* on another hand, the application of the water fern can be feasible for commercially β-carotene purification.

Keywords: *Azolla*, β-carotene, Column chromatography, HPLC, Natural product

*Corresponding Author: **Ghorban Ali Nematzadeh**
Tel.: +9809121328337

Introduction

β-carotene and other carotenoid pigments are the natural product of plants including algae with pro-vitamin A activity and have a significant role in human health for example natural antioxidants, protecting cells from oxidative harmful effects of singlet oxygen and free radicals [1, 2]. Many reports are indicating sturdy correlations among carotenoid consumption and decreased danger of bone calcification, such as atherogenesis, cancer, eye degeneration, neuronal damages, and bone calcification [3]. Many types of research have demonstrated increase inhibition of cancer cells and tumor regression in animals fed diets that are rich in carotenoids [4]. Hence, carotenoids, particularly β-carotene, are broadly utilized by the food, pharmaceutical, and cosmetic industries. Increasing demand for β-carotene, frequently natural β-carotene, has led to rising consideration in extracting β-carotene from various natural sources for example vegetables and algae [1].

Azolla is a free-floating water fern that belongs to the *Azollaceae* family [5, 6]. *Azolla* has numerous usages, for instance, animal feed, human food, medicine, production of biogas, hydrogen fuel, water purifier, weed control, bio-fertilizer, and reduction of ammonia volatilization and is competently mentioned as green gold mine [5, 7]. *Azolla* is distributed on a wide scale in temperate and tropical regions [8]. The genus is classified into two sub-genera *Euazolla* and *Rhizosperma*. *Euazolla* involves five novel world species namely *A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla*, and *A. rubra* and subgenus *Rhizosperma* contain two old world species namely *A. pinnata* and *A. nilotica* [8]. Azolla is a rich source of proteins, essential amino acids, vitamins (vitamin A, vitamin B12, β-carotene), growth promoter intermediaries, and inorganics such as phosphorous, calcium, potassium, magnesium, copper, ferrous, and so on [5].

Stancher et al., (1988) [9] reported the chromatographic profiles of carotenoids from some marine shellfish. The analyses were carried out both by gradient elution reversed-phase and isocratic direct phase high-performance liquid chromatography (HPLC). The six major carotenoids extracted from three shellfish species (*Chlumys opercularis* L., *Cardium tuberculatum* L., *Pecten jacobaeus* L.) in extremely pure form using semi-preparative HPLC. Craft et al., (1992) [10] monitored changes in separation seven-component carotenoid mixture selectivity in response to variations in HPLC conditions. Resolution of lutein/zeaxanthin and β-carotene/lycopene were better at lower temperatures whilst echinenone/α-carotene separation

improved as temperature increased. Lejeune et al., (2000) [11] analyzed the carotene content of six *Azolla* strains under greenhouse and growth chamber conditions. On fresh substance, the carotene content ranged from 206 to 619 mg kg^{-1} on a dry matter basis and differed considerably among strains. In both culture conditions, *Azolla filiculoides* 173F1 and *Azolla mexicana* 220 ME were the richest and poorest source of carotene, respectively. Kumar and Sinha (2004) [12] discussed natural colorant sources, chemical constituents responsible for producing different colors, and their activities. Taylor et al., (2006) [13] used a reverse-phase C30 column with a binary mobile solvent system for the baseline separation of eight of the major carotenoids and the two chlorophylls (a and b) inside 18 min. These compounds were recognized through the utilization of authentic standards, their spectral characteristics, and HPLC- atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) confirmation. Abu-Rezq et al., (2010) [14] carried out the β-carotene extraction of *Dunaliella salina* using the pressured fluid extraction method. The obtained results presented that *D. salina* samples included comparatively good amounts of β-carotene 33.8-96.5 pg $cell^{-1}$. Ullah et al., (2011) [15] investigated the content of β-carotene in bath spongy, bitter guard, bottle guard, bringal, cabbage, carrot, cucumber, french beans, green chili, khulfa, lady finger, lettuce, mushrooms, mint, mountain ebony, onion, potato, red chili, spinach and tomato by HPLC. The β-carotene content differed from trace amount in potato, mushroom, and mountain ebony to thousands μg 100 g^{-1} in carrot, spinach, mint, and lettuce. Mostafa and Ibrahim (2012) [16] placed *Azolla caroliniana* under UV-B radiation for 24 and 48 h. The results indicated that prolonged UV-B exposure of *A. caroliniana* for 48 h resulted in a decrease in peaks area for β-carotene. Rebecca et al., (2014) [17] presented that carrot, red spinach, red capsicum, yellow capsicum, beetroot and broccoli with 18.3, 5.6, 2.4, 2.4, 1.9 and 1.3 mg 100 g^{-1} samples have the most amount of β-carotene respectively. Chandra-Hioe et al., (2017) [18] determined β-carotene content in Chinese broccoli, Chinese white cabbage, Chinese spinach, coriander, water spinach, and Chinese mustard, by HPLC. The β-carotene content was 6604±963, 4142±198, 2546±191, 2524±776, 2460±459, 2446±225, 1974±549, respectively. Gharbi et al., (2017) [19] showed that Tunisian tomato peels and seeds can be a potential source of natural bioactive compounds. Jin et al., (2017) [20] developed a simple ultra-high- pressure liquid chromatography (UHPLC) technique for rapidly and simultaneously recognizing thirteen carotenoids in *Haematococcus pluvialis*. Noormohammadi (2019) [25] investigated the drilling flowers, types of contaminations and ways to treat them. Beiraghi et al., (2019) [26] studied the

separation, preconcentration, and determination of Hg (II) ion in water samples by cloud point extraction technique coupled with UV-VIS spectrophotometry using a new complexing agent. Darouneh and Zargan (2018) [27] produced biofertilizer from gas refinery wastewater. Dejene et al., (2020) [28] determined the selected pesticide residues from Gilgel Gibe (I) hydroelectric dam reservoir and its tributaries, Jimma Zone, Ethiopia. Beiraghi and Roshdi (2018) [29] evaluated selective separation and preconcentration of trace amounts of Gallium in water and rice samples using cloud point extraction and determination by inductively coupled Plasma-Atomic Emission Spectrometry. Benhachem et al., (2019) [30] investigated Kinetic adsorption of methylene blue dye from aqueous solutions using activated carbon from starch. Oyibo et al., (2019) [31] evaluated the use of strawberry and Arabic gum blend as an inhibitor for the corrosion of aluminum in an acidic medium. Rostamoghli et al., (2018) [32] Applied the $B_{12}N_{12}$ nanoparticle as the CO, CO_2, H_2O and NH_3 sensor.

The purpose of this research was to extract, purify and identify β-carotene from *Azolla Pinnata* R. Br.

Experimental

Preparation of sample

Azolla pinnata R. Br. was collected on August 2018 from genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) experimental farm located at 53^{o} 4″ E and 36^{o} 39″ N and divided into two equal portions. One portion was a fresh sample that was quickly frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle and the other portion was shade-dried and pulverized to powder in a mechanical grinder.

Solvent extraction and isolation of β-carotene

Seven gram of fresh and dried *A. pinnata* R. Br. powder separately were mixed with 100 mL of Hexane/Acetone solution (60:40 v/v) including 0.1% butylated hydroxyl toluene (BHT) on a rotational suspension mixer for 30 min at 45 rpm before centrifugation and separation of the extract. Each sample was extracted three times to obtain a colorless residue, and then extracts were pooled together. The AOAC (Association of official analytical chemists) method (1990) [21] was applied for the saponification of pigments. Saponified extract transferred to a separatory

funnel and then added 10% (w/v) Na_2SO_4. The bottom layer removed and the upper layer washed three times with distilled water. 3 g anhydrous sodium sulfate added and filtered. Finally, the upper layer including the lipophilic carotenoids concentrated in a rotary evaporator attached to a vacuum pump at 40 ^oC.

Purification of β-carotene using column chromatography

The bottom of a chromatography column was plugged with glass wool. The column was mounted on a vacuum filtration device by a filtration flask as a receiving container. Silica gel 60 GF254 (Merck) Added to obtain a 20 cm layer while using a vacuum. Leveled the surface of the silica gel and placed a film 2 cm layer of anhydrous Na_2SO_4 on top. The carotenoid was fractionated by various ratios of Hexane/Acetone (100:0; 99:1; 98:2; 97:3; 96:4; 0:100) [22].

Characterization of β-carotene

Preparation of β-carotene standard

β-carotene (>93% UV) standard was obtained from Sigma-Aldrich. A stock solution (1 mg mL^{-1}) was prepared with Hexane/Acetone + 0.1% BHT and working standards (10-200 µg mL^{-1}) were made using serial dilution before spectrophotometry and chromatography.

Quantification of β-carotene content by HPLC

The β-carotene content was assessed by HPLC (Knauer, Advanced scientific instruments, Germany), by C_{18} column (250 × 4.6 mm internal diameter × 5 µm film thickness). The HPLC conditions were: (a) mobile phase (Methanol/THF/water; 67:27: 6), (b) flow rate of 1.0 mL min^{-1}, (c) injection volume of 20 µL, (d) run time of 30 min, (e) column temperature of 35 ^oC, (f) variable wavelength UV/Vis detector at λ_{max}= 450 nm.

Spectroscopic data of β-carotene

The β-carotene was analyzed using FT-IR spectroscopy (Bruker vector in KBr matrix), ^1H, and ^{13}C NMR spectra (BRUKER DRX-400 AVANCE spectrometer).

Results and discussion

Purification of β-carotene

From all solvents ratio examined, Hexane/Acetone 96:4 (v/v) indicated the best results (obtaining the characteristic spectral profile of these compounds), with a virtually complete extraction of β-carotene, while with other ratios of solvents other carotenoids were separates.

Comparing the amount of β-carotene extracted from dried and fresh Azolla Pinnata R. Br.

The results of the amount of β-carotene extracted from fresh and dried *A. Pinnata* R. Br. indicated that the amount of β-carotene extracted from fresh *A. Pinnata* R. Br. is approximately twice of dried *A. Pinnata* R. Br.

Optimization of HPLC method

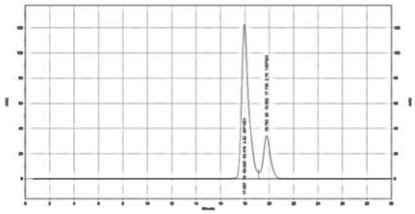
The mixture of Methanol/THF/water; 67:27:6 (v/v) as mobile phase showed the best resolution for β-carotene (calculated as the ratio between the difference in retention times and the average of the width of the peaks at half-maximum height) (Table 1). The selected mobile phase provided good retention factors (4.03), a high number of plates, and tailing factors of 1 for both compounds studied. In these conditions, a resolution of 3.75 was obtained.

Table 1. Chromatographic parameters of β-carotene using different mobile phases.

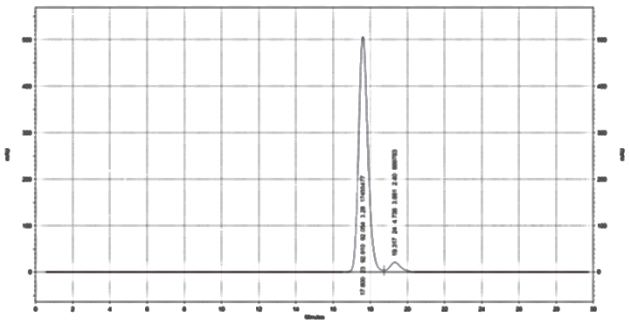
Mobile phase	P′	R	α	N	β-carotene	
					K′	T
Methanol/THF/water; 67:27:6 (v/v)	**5.17**	**3.75**	**1.29**	**2004.47**	**4.03**	**1.00**
Methanol/THF/water; 67:27:6 (v/v)	**5.08**	**2.09**	**1.23**	**1182.76**	**2.63**	**1.33**
Methanol/THF/water; 67:27:6 (v/v)	**5.23**	**2.22**	**1.18**	**1668.07**	**4.48**	**0.83**
Methanol/THF/water; 67:27:6 (v/v)	**5.21**	**2.79**	**1.16**	**2506.22**	**6.67**	**1.00**
Methanol/THF/water; 67:27:6 (v/v)	**5.09**	**2.13**	**1.24**	**1394.18**	**4.47**	**1.25**

MeOH: methanol; P′: polarity index; R: resolution; α: selectivity; N: number of plates; T: tailing factor; K′: retention factor

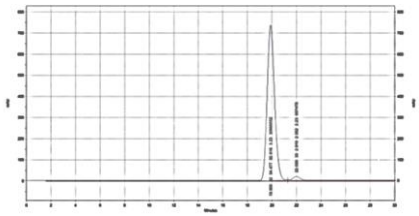
The HPLC method was applied to β-carotene-rich *A. Pinnata* R. Br. and the UV-Vis spectra of the samples were similar to the standard β-carotene curve (Figure 1). As is clear from the chromatograms, fresh *A. Pinnata* R. Br. is in the concentration range of 20 to 50 ppm, and dried *A. Pinnata* R. Br. is in the concentration range of about 20 ppm for seven gram of primary samples.



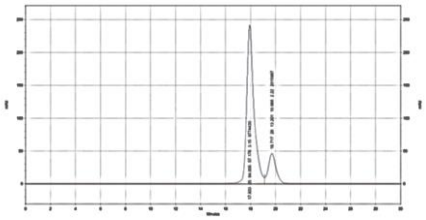
Chromatogram of working standard solution at 20 ppm concentration



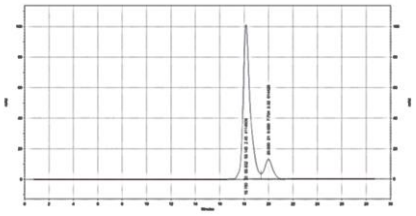
Chromatogram of working standard solution at 50 ppm concentration



Chromatogram of working standard solution at 100 ppm concentration



Chromatogram of fresh *Azolla*



Chromatogram of dried *Azolla*

Figure 1. Chromatogram of working standard solutions and fresh and dried *Azolla*

Spectroscopic data of β-carotene

Figure 2 displays the FT-IR spectrum of β-carotene. The β-carotene spectrum showed peaks at 2919.63 cm^{-1} and 2859.47 cm^{-1} for asymmetric and symmetric stretching vibrations of the CH_2 and CH_3, 1448.74 cm^{-1} for CH_2 scissoring, 1363.69 cm^{-1} for splitting due to dimethyl group, 1010.28 cm^{-1} for in plane –CH- and 966.31 cm^{-1} for trans conjugated alkene –CH=CH– out of plane deformation mode. Also, the ^{1}H and ^{13}C NMR spectra of β-carotene are shown in Figures 3 and 4. Furthermore, spectroscopic data for β-carotene follow:

^{1}H NMR (400 MHZ, $CDCl_3$): δ = 6.70-6.63 (m, 2H), 6.37 (d, *J* = 7.4 HZ, 1 H), 6.27 (d, *J* = 5.6 HZ, 1 H), 6.18-6.12 (m, 3H), 2.04 (d, *J* = 6 HZ, 2 H), 1.99 (s, 6 H), 1.74 (s, 3 H), 1.65-1.62 (m, 2 H), 1.50-1.47 (m, 2 H); ^{13}C NMR (100 MHZ, $CDCl_3$): δ = 137.92, 137.77, 137.24, 136.48, 136.03, 132.43, 130.85, 129.99, 129.39, 126.66, 125.04, 39.65, 34.28, 33.12, 28.98, 21.78, 19.27, 12.83, 12.77.

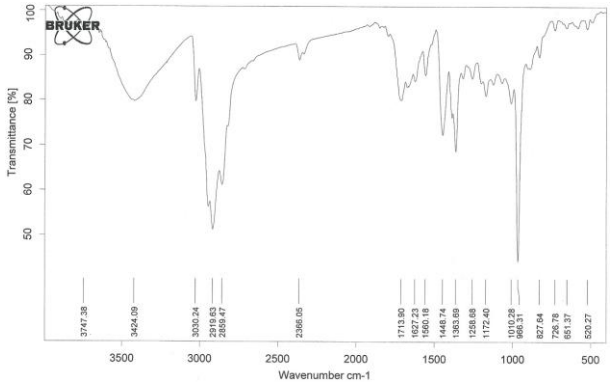


Figure 2. FT-IR spectrum of β-carotene

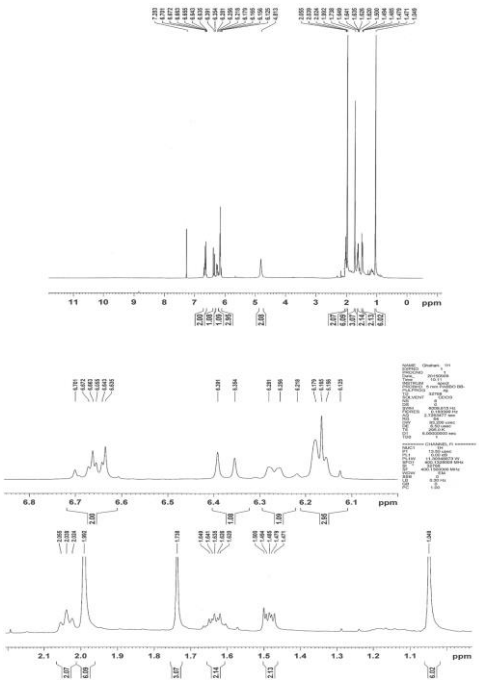


Figure 3. ^{1}H NMR spectra of β-carotene

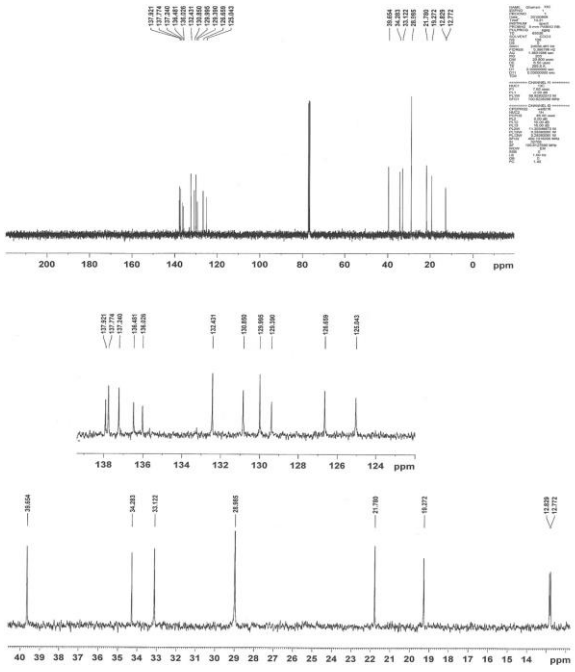


Figure 4. ^{13}C NMR spectra of β-carotene

Conclusion

Many vegetables, fruits, and algae such as carrot, *Dunaliella sp.* and, etc. known as wealthy sources of β-carotene [23], but the production and maintenance require consuming a lot of time and money. All these problems were caused by the researchers are looking for other new carotenoid wealthy sources that easily available and low costs of production and maintenance be cheap. *Azolla* is a wealthy source of β-carotene [11], which can be considered as a weed in rice fields and does not require special storage conditions for growth. This floating water fern is naturally growing in the fresh pound, marsh and rice fields in the northern part of Iran, especially in spring and summer. So, its production is free to coast and can be a suitable source of β-carotene. The *Azolla* reproduction cycle is short and can be double in during 3-5 days [24]. Also, this plant can replicate in all seasons, and while many carotenoids rich plants are not able to grow and multiply in all seasons. These advantages caused in this research, the extraction, isolation, and purification of β-carotene considered from this worthy weed.

Acknowledgments

The financial support of this work from Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University is gratefully acknowledged.

References

[1] M. Hejazi, C. De Lamarliere, J. Rocha, M. Vermue, J. Tramper, R. Wijffels, Biotechnol. Bioeng., 79, 29, (2002).

[2] P. Karnjanawipagul, W. Nittayanuntawech, P. Rojsanga, L. Suntornsuk, Warasan Phesatchasat, 37, 8, (2010).

[3] A.O. Barba, M.C. Hurtado, M.S. Mata, V. F. Ruiz, M.L.S. De Tejada, Food Chem, 95, 328, (2006).

[4] M.M. Barth, C. Zhou, K.M. Kute, G.A. Rosenthal, J. Agric. Food Chem., 43, 2876, (1995).

[5] K. Bindhu, Int. res. j. biol. sci., 2, 88, (2013).

[6] W. Raja, P. Rathaur, S.A. John, P.W. Ramteke, Hacettepe J. Biol. Chem., 40, 1, (2012).

[7] R. Yadav, G. Abraham, Y. Singh, P. Singh, in 'Advancements in the utilization of Azolla-Anabaena system in relation to sustainable agricultural practices', 2014.

[8] W. Raja, P. Rathaur, S.A. John, P.W. Ramteke, Int. J. Res. Biol. Sci., 2, 68, (2012).

[9] B. Stancher, F. Zonta, L.G. Favretto, J Chromatogr A, 440, 37, (1988).

[10] N.E. Craft, S.A. Wise, J.H. Soares Jr, J Chromatogr A, 589, 171, (1992).

[11] A. Lejeune, J. Peng, E. Le Boulengé, Y. Larondelle, C. Van Hove, Anim. Feed Sci. Technol. 84, 295, (2000).

[12] J. Kumar, A.K. Sinha, Nat. Prod. Res., 18, 59, (2004).

[13] K.L. Taylor, A.E. Brackenridge, M.A. Vivier, A. Oberholster, J Chromatogr A, 1121, 83, (2006).

[14] T.S. Abu-Rezq, S. Al-Hooti, D. Jacob, M. Al-Shamali, A. Ahmed, N. Ahmed, Algal Biomass Utln, 1, 58, (2010).

[15] N. Ullah, A. Khan, F.A. Khan, M. Khurram, M. Hussan, S.M. U. Khayam, J. Hussain, Middle East J. Sci. Res., 9, 496, (2011).

[16] E. Mostafa, M. Ibrahim, Egypt. Acad. J. Biolog. Sci., 3 (1), 19, (2012).

[17] J. Rebecca, S. Sharmila, M.P. Das, C. Seshiah, J. Chem. Pharm. Res., 6, 594, (2014).

[18] M. Chandra-Hioe, H. Rahman, J. Arcot, J Food Chem Nanotechol, 3, 93, (2017).

[19] S. Gharbi, G. Renda, L. La Barbera, M. Amri, C. Messina, A. Santulli, Nat. Prod. Res., 31, 626, (2017).

[20] H. Jin, Y.M. Lao, J. Zhou, H.J. Zhang, Z.H. Cai, J Chromatogr A, 1488, 93, (2017).

[21] AOAC (Association of Official Analytical Chemists) Carotenes and Xanthophylls in dried plant materials and mixed feeds. AOAC method 970.64. In: Helrich K (ed) AOAC official methods of analysis, 15th edn. AOAC, Arlington, 1048-1049, (1990).

[22] S. Machmudah, M. Goto, in 'Methods for extraction and analysis of carotenoids', Springer, (2013).

[23] H. Rymbai, R. Sharma, M. Srivastav, Int. J. PharmTech Res, 3, 2228, (2011).

[24] G.M. Wagner, Bot. Rev., 63, 1, (1997).

[25] M. Noormohammadi, Int. J. New. Chem, 6, 14-22, (2019).

[26] A. Beiraghi, A.R. Shirkhani, A. Ziyaei Halimehjani, Int. J. New. Chem, 6, 163-177, (2019).

[27] E. Darouneh, M. Zargan, Int. J. New. Chem, 5, 493-499, (2018).

[28] M. Dejene, K. Kedir, S. Mekonen, A. Gure, Int. J. New. Chem, 7, 14-29, (2020).

[29] A. Beiraghi, M. Roshdi, Int. J. New. Chem, 5, 124-139, (2018).

[30] F.Z. Benhachem, T. Attar, F. Bouabdallah, *Chem Rev Lett*, 2, 33-39, (2019).

[31] D.E. Arthur, *Chem Rev Lett*, 2, 69-75, (2019).

[32] R. Rostamoghli, M. Vakili, A. Banaei, E. Pourbashir, K. Jalalierad, *Chem Rev Lett*, 1, 31-36, (2018).

HOW TO CITE THIS ARTICLE

Sajjad Ghahari, Somayeh Ghahari, Ghorban Ali Nematzadeh, "**Isolation, Purification and Identification of β-carotene from Azolla Pinnata R. Br. as a New Carotenoid Wealthy Source"** International Journal of New Chemistry., 2021; DOI: 10.22034/ijnc.2021.122084.1098