Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
an open access publisher
http://ansinet.com

OPEN ACCESS Asian Journal of Plant Sciences

ISSN 1682-3974
DOI: 10.3923/ajps.2022.192.202

Research Article
In vitro Micropropagation, Biological Activities and Phenolic Profile of *Astragalus fruticosus* Forssk

[1]Rawia A. Zayed, [2]Ashraf S. El-Sayed and [1]Wafaa M. Ismaeil

[1]Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, 44519, Egypt
[2]Department of Botany and Microbiology, Faculty of Science, Zagazig University, Zagazig, 44519, Egypt

Abstract

Background and Objective: *Astragalus fruticosus* Forssk. is an endangered wild Egyptian plant, with a poor germination ratio and rich in valuable phytochemicals. This study was aimed to develop an efficient micropropagation protocol as well as a screening of the cytotoxic, antidiabetic activities and phenolic profile of the micropropagated plantlets. **Materials and Methods:** Murashige and Skoog (MS) medium fortified with different concentrations of N^6-Benzyl Amino Purine (BAP), Kinetin (Kn) and thidiazuron (TDZ) either singly or with auxins as 2, 4-Dichlorophenoxyacetic acid (2,4 D) was used for regeneration studies. Cell viability assay and α-glucosidase inhibition methods were used to evaluate cytotoxic and antidiabetic activities, respectively. **Results:** MS medium supplemented with 1 mg L^{-1} BAP was optimum for direct shoot regeneration from explants. Regenerated shoots were rooted and complete plantlets were obtained and successfully acclimatized and grown under greenhouse conditions. Embryogenic callus was induced in MS medium with 1 mg L^{-1} 2,4 D showing 85.3% callusing capacity, small plantlets of 1.3-2.4 cm height were regenerated. Micropropagated plants showed strong cytotoxic and significant antidiabetic activities. **Conclusion:** Somatic embryogenesis and direct organogenesis are efficient regeneration systems that might be suitable for further biotechnological approaches as genetic transformation and somatic hybridization for improvement of seed germination ratios of this plant species.

Key words: *Astragalus fruticosus* Forssk., organogenesis, embryogenic callus, plant regeneration, cytotoxicity, antidiabetic activity, phenolics

Citation: Zayed, R.A., A.S. El-Sayed and W.M. Ismaeil, 2022. *In vitro* micropropagation, biological activities and phenolic profile of *Astragalus fruticosus* Forssk. Asian J. Plant Sci., 21: 192-202.

Corresponding Author: Rawia A. Zayed, Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, 44519, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Astragalus L. is the largest genus in the family Fabaceae, it has been estimated to include about 2000-3000 species and about 250 taxonomic sections in the world[1,2]. In Egypt, Allam *et al.*[3] reported that the genus *Astragalus* is represented by thirty-five species, while Radwan *et al.*[4] reported thirty-two species. Phytochemical investigation on this genus revealed that it is a rich source of important medicinally active secondary metabolites as bioactive saponins[5,6], flavonoids[7] and polysaccharides[8] with a variety of effects as immunomodulatory[9], hepatoprotective[3], cytotoxic[10] and antioxidant[11].

A huge number of plants species are becoming lost irreversibly because of both the destruction of their habitats by increasing the expansion in construction by the human population as well as over-collection for their valuable phytochemicals. Then the *in vitro* micropropagation and regeneration system is considered as a potential alternative to induce the biomass plants tissues and bioactive natural compounds[12-16]. Reports regarding *Astragalus* species regeneration systems are very few[17].

To date, no progress has been made for developing an *in vitro* regeneration system for *Astragalus fruticosus* Forssk.

In this study, an efficient protocol for micropropagation of *Astragalus fruticosus* via organogenesis is developed for the first time to produce plant materials for secondary metabolites studies and to save this medicinally valuable plant from extinction. Moreover, cytotoxic and antidiabetic effects of the *in vitro* micropropagated plantlets were evaluated for the first time. Phenolic compounds can significantly reduce the risk of cardiovascular diseases, stroke and certain types of cancer[18].

Therefore, total phenolic and flavonoid contents of the callus from medium II and the *in vitro* cultured plantlets ethanolic extracts were estimated for the first time.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt from June, 2018-September, 2020.

Plant material: *Astragalus fruticosus* seeds were collected from North Sinai, Egypt (between latitude 31° 07′ 09.5″ N and longitude 33°41′ 57.1″ E) in April, 2018. The plant identification and verification were kindly performed by Prof. Hussein A. Hussein, Professor of Plant Taxonomy and Flora, Department of Botany and Microbiology, Faculty of Science, Zagazig University, Egypt.

Seeds sterilization and germination: Mature seeds of *Astragalus fruticosus* were surface-sterilized through washing with 70% ethyl alcohol for different periods (1, 3 and 5 min) then shaking with 5% hypochlorite solution for different periods (3, 5 and 15 min). Seeds were then rinsed 3 times with sterile double distilled water for 2 min. *A. fruticosus* has poor seed germination capacity due to hard seed coat so, mechanical scarification was so helpful. Scratched and non-scratched seeds were cultivated over Murashige and Skoog (MS) medium (4.4 g L^{-1}, Duchefa, Germany), with 3% sucrose (Adwick, ARE) either in liquid form or solidified with 0.8% agar (Bioworld, USA), either without any phytohormones or supplemented with different concentrations of phytohormones and Gibberellin A_3 (GA_3). Moreover, 6-8 seeds were inoculated into sterile petri-dish with sterile moistened filter paper. PH was adjusted to 5.6-5.8. Jars were incubated at 25°C under a white fluorescent lamp (16/8 hrs photoperiod). Seed Germination was investigated using seed germination percentage, 4 weeks after cultivation.

Callus induction: Explants (0.5-1 cm) were excised aseptically from the stem and leaf of 28-day-old seedlings. Then cultured in jars containing MS media with 30 g L^{-1} sucrose, 8 g L^{-1} agar and supplemented with different concentrations of plant growth regulators.

Medium I: 1 mg L^{-1} 2, 4-D, Medium II: 0.1 BAP+1 mg L^{-1} 2, 4-D+0.5 mg L^{-1} TDZ, Medium III: 2 mg L^{-1} 2, 4 D+1 mg L^{-1} Kinetin, Medium IV: 0.5 mg L^{-1} BAP, Medium V:1 mg L^{-1} BAP, Medium VI: 0.5 mg L^{-1} TDZ, Medium VII: 1 mg L^{-1} Kinetin and Medium VIII: 1 mg L^{-1} NAA+0.1 mg L^{-1} BAP. PH was adjusted to 5.6-5.8 and jars were maintained in the dark at 25°C. The best medium was selected by determination of callusing capacity and callus dimensions.

Growth dynamics: The greatest callusing capacity was observed on medium II providing healthy calli with the largest dimensions so, it was chosen to study the growth dynamics.

Growth curve: To characterize the callus growth in medium II, a growth curve was established. One gram of 5 weeks old calli was subcultured on the same medium, cultures were maintained at 25°C in the dark. Fresh and dry weights were

measured every 3 days for 30 days and the mean values of triplicate readings were plotted against time to obtain the growth curve[19,17]:

$$\text{Growth index (GI)} = \frac{\text{Ge} - \text{Gstart}}{\text{Gstart}}$$

where, Ge is callus weight at the end of generation (final fresh weight), G start is callus weight at zero time (Initial fresh weight). Relative Growth Rate (RGR) was determined on fresh weight using the following Eq.[20]:

$$\text{RGR} = 3\ \frac{\text{Wf}_{1/3} - \text{Wi}_{1/3}}{\text{tf-ti}}$$

where, ti is Beginning of the subculture, tf is Final day of subculture, after 30 days, Wi is Initial Weight of calli biomass (at ti), Wf is Final weight of calli biomass (at tf), tf-ti is 30 days of subculture.

Specific growth rate (μ):

$$\mu = \frac{\ln x - \ln xo}{t}$$

where, xo is the initial weight of dry biomass and x is the biomass weight at time t (30 days)[19].

Doubling time is the required time for doubling of biomass of cells and is denoted as (dt). It can be determined by the following Eq.:

$$dt = \ln \frac{2}{\mu}$$

where, μ is the specific growth rate[19].

Shoot induction: Explant segments (0.5-1 cm) were aseptically dissected from 28-day-old seedlings, then aseptically cultured in jars containing MS media with 30 g L^{-1} sucrose, 8 g L^{-1} agar and supplemented with various concentrations of phytohormones, Medium IV: 0.5 mg L^{-1} BAP, Medium V: 1 mg L^{-1} BAP, Medium VI: 0.5 mg L^{-1} TDZ, Medium VII: 1 mg L^{-1} Kinetin and Medium VIII: 1 mg L^{-1} NAA+0.1 mg L^{-1} BAP. PH was adjusted to 5.6-5.8 and jars were incubated at 25°C under a white fluorescent lamp with a 16/8 hrs light/dark period. The most appropriate medium for shoot induction was selected by determination of the number of explants produced shoots and the number of produced shoots per explants after the third subculture.

In vitro* propagation of *Astragalus fruticosus

Regeneration of *Astragalus fruticosus* plantlet via somatic embryogenesis: Three months-old nodular embryogenic calli grown on Medium I and Medium II were aseptically transferred to solid MS media with the same hormonal composition with 8 g L^{-1} agar and 30 g L^{-1} sucrose. Then they were kept at room temperature, in a dark condition. The cultures were maintained at 25°C in the dark.

Regeneration of *Astragalus fruticosus* plantlet via organogenesis: Two months-old shoots (4-8 cm) from Medium V were transferred individually into jars containing half-strength rooting media consisting of ½MS with 30 g L^{-1} sucrose, 0.6% agar and 1 mg L^{-1} NAA. Cultures were incubated at 25°C under a white fluorescent lamp with a 16/8 hrs photoperiod.

Acclimatization of the *in vitro* regenerated plantlets: Healthy regenerated plantlets were removed from their culture vessels, gently washed under running tap water then transplanted into plastic pots containing sterile soil: sand (1:1) under 16/8 hrs photoperiod and covered with perforated transparent plastic bags which were removed after one week. Pots were maintained in the greenhouse.

Cytotoxic activity: MCF-7 (human breast cancer) cell line and HL-60 (human promyelocytic Leukemia carcinoma) cell lines (VACSERA, Egypt) were used to evaluate the cytotoxicity of the micropropagated plantlet alcoholic extract at different concentrations (500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 μg) using cell viability colorimetric assay[21] and cisplatin as a standard. The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as:

$$\frac{\text{ODt}}{\text{ODc}} \times 100\ (\%)$$

where, ODt is the mean optical density of cells treated with a tested sample and ODc is the mean optical density of untreated cells. The survival curve of each cell line was constructed. IC_{50} was estimated[22].

Antidiabetic activity: α-glucosidase inhibitory activity of varying concentrations (1000 - 7.81 μg mL^{-1}) of ethanolic extract of *in vitro* regenerated plantlets was carried out according to the method reported by Shai *et al.*[23]. The absorbance was measured at 405 nm using Multiplate

Reader. Acarbose was included as a standard. The results were expressed as percentage inhibition, using the formula:

$$\text{Inhibitory activity (\%)} = \frac{1 - As}{Ac} \times 100$$

where, As is the absorbance of the reaction mixture in the presence of the tested sample and Ac is the control absorbance. The IC_{50} was estimated[24].

Estimation of total phenolic content: Spectrophotometric estimation of total phenolic content in the ethanolic extracts of callus and *in vitro* cultured plant was performed using the Folin-Ciocalteu colorimetric methods [25]. The absorbance was measured using the spectrophotometer at 765 nm. Gallic acid was used as a standard (50-300 µg mL^{-1}) in 95% ethanol. Phenolics concentration (mg mL^{-1}) in the extracts was deduced from the gallic acid calibration curve and expressed as gallic acid equivalent (mg of GA g^{-1} extract).

Estimation of total flavonoid content: Spectroscopic determination of total flavonoids content in callus and *in vitro* cultured plant extracts was carried out using aluminium chloride colorimetric method[26]. The absorbance of the mixture was measured at λmax 415 nm (using a UV/Vis spectrophotometer). Quercetin was used as a standard, the standard curve was plotted (50-300 µg mL^{-1}) in 95% ethanol. Total flavonoid contents of the tested extracts were expressed as quercetin equivalent (mg of QU g^{-1} of the extract).

Analysis of extracts by High-Performance Liquid Chromatography (HPLC): Phenolic and flavonoid components of the ethanolic extracts of callus and *in vitro* cultured plant were studied using HPLC (Agilent1100 series) using standard phenolic acids (caffeic, gallic, protocatechuic, ellagic, syringic, ferulic, ascorbic, chlorogenic, *p*-coumaric, eugenol and pyrogallol) in addition to standard flavonoids as rutin, naringenin, apigenin, luteolin, kaempferol, 3-hydroxy flavone and myricetin (Sigma®) according to the method reported by Zuo *et al*.[27].

Standard curves were established (10, 20, 40, 60, 80, 100 and 200 mg L^{-1}). Phenolics in tested samples were detected (UV detector) through a gradient elution of the mobile phase. Chromatograms were recorded at 255 nm for luteolin, kaempferol, 3-hydroxy flavone and rutin, 340 nm for apigenin, 370 nm for myricetin and 325 nm for caffeic acid, syringic acid and other phenolics.

RESULTS AND DISCUSSION

Seeds decontamination rate and seeds germination capacity: The best seed sterilization condition with 100% germination was achieved by exposure of the seeds to 70% ethanol for 5 min then 5% sodium hypochlorite for 15 min. Maximum seeds germination percentage, (100%) was achieved on the second day of culture in all culture media and conditions only for scratched seeds as shown in Fig. 1a-e and Table 1. While there was no sign of seed germination was observed for non-scratched seeds, (0% germination). Seed dormancy is common in most Fabaceae species due to the hard seed coat that blocks water passage to the embryo[28]. Mechanical scarification of *A. fruticosus* seeds was a vital step for seed germination. Seed coat incision in *Astragalus cariensis* Bioss. Erisen *et al*.[29] provided successful germination percentages similar to our results.

Callus induction: Explants cut ends showed the earliest sign of callus formation after 2 weeks of culture then spread towards the middle region of explants. The effect of phytohormones on the *in vitro* induced calli and the callusing capacity as well as, the morphological characters on different media after 5 weeks of cultivation were shown in Fig. 2a-e and Table 2. Medium II showed the best

Table 1: Germination percentage of *A. fruticosus* seeds

		Germination (%)	
Media	Phytohormones	Scratched seeds	Non-scratched seeds
Liquid MS	No phytohormones	100	No germination
Liquid MS	50 mg L^{-1} GA_3	100	No germination
Liquid MS	100 mg L^{-1} GA_3	100	No germination
Liquid MS	150 mg L^{-1} GA_3	100	No germination
Solid MS	No phytohormones	100	No germination
Solid MS	1 mg L^{-1} BAP	100	No germination
Solid MS	1 mg L^{-1} 2,4-D	100	No germination
Solid MS	0.1 mg L^{-1} BAP+1 mg L^{-1} 2, 4-D+0.5 mg L^{-1} TDZ	100	No germination
Filter paper moistened with sterile dist.H_2O	No phytohormones	100	No germination

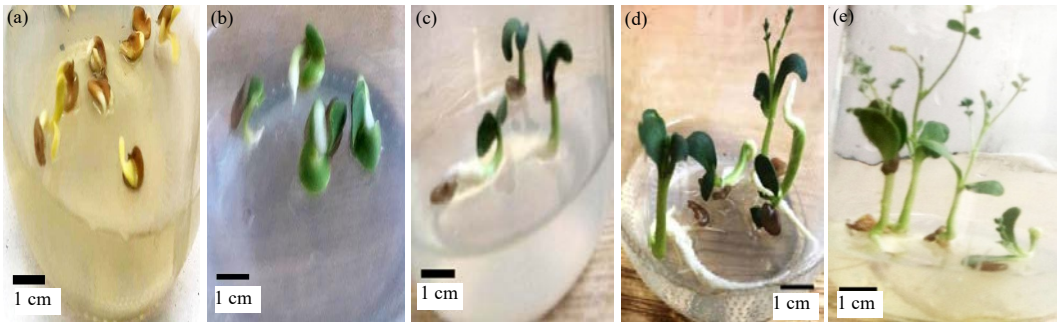

Fig. 1(a-e): Seed germination (a) One day old seedling, (b) Three days old seedling, (c) One-week old seedling, (d) Two weeks old seedling and (e) Four weeks old seedling (bar 1 cm, a-e)

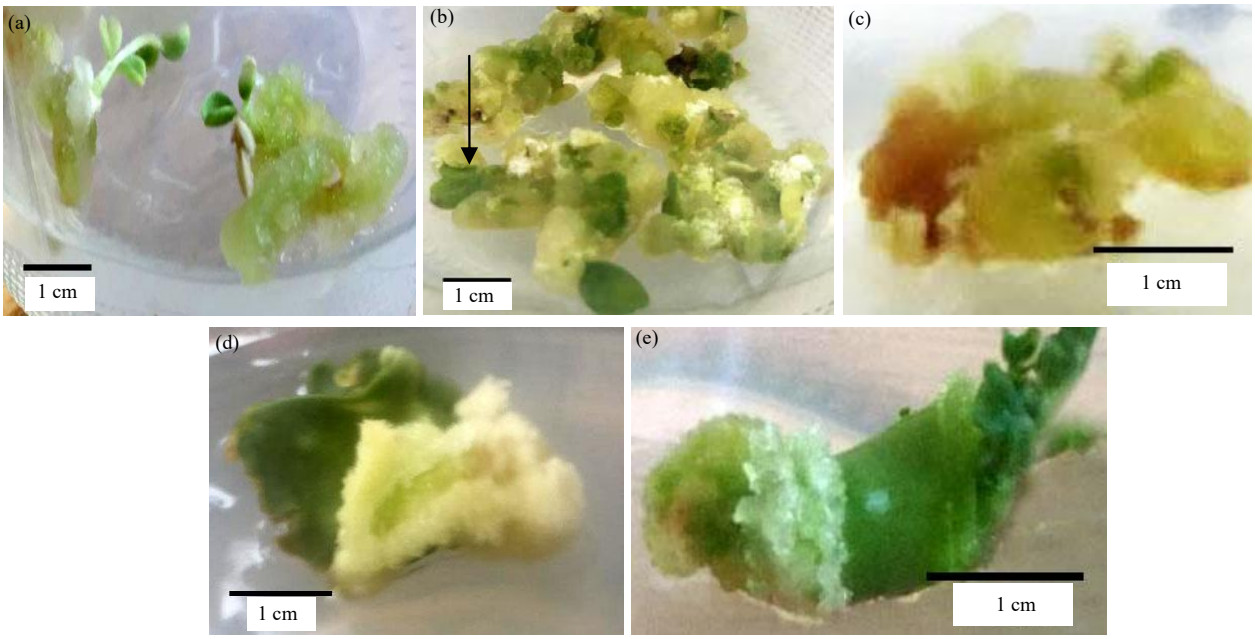

Fig. 2(a-e): Callus induction in different media, (a) Callus on medium I with cotyledonary leaves, (b) Callus on medium II with cotyledonary leaves, (c) Callus on medium III, (d) Callus on medium IV and (e) Callus on medium V (bar 1 cm, a-e)

Table 2: Morphological characteristics and dimensions of the callus from seedling explants

Medium	Morphological characteristics	Dimension	Callusing capacity (%)
I	Yellowish green, friable	+++	85.3
II	Yellowish green, compact	++++	100.0
III	Yellowish white, friable	++	45.7
IV	Yellowish white, friable, small in diameter with direct shoot formation	+	10
V	Yellowish white, friable, very small in diameter with direct shoot formation from explants	+	6.2
VI	No sign of callus formation but direct shoot formation from explants	---	0.0
VII	No sign of callus formation but direct shoot formation from explants	---	0.0
VIII	No sign of callus formation but direct shoot formation from explants	---	0.0

+: Very weak growth (2-5 mm in diameter), ++: Moderate growth (5-10 mm in diameter), +++: Good growth (10-15 mm in diameter) and ++++: Very good growth (20-25 mm in diameter)

callusing capacity so, it was chosen for further investigations. It has never been reported before in any literature for the genus *Astragalus*.

Growth parameters: The growth curve for 5 weeks old callus grown on medium II as shown in Fig. 3 was established depending on fresh and dry weight measurement. It showed

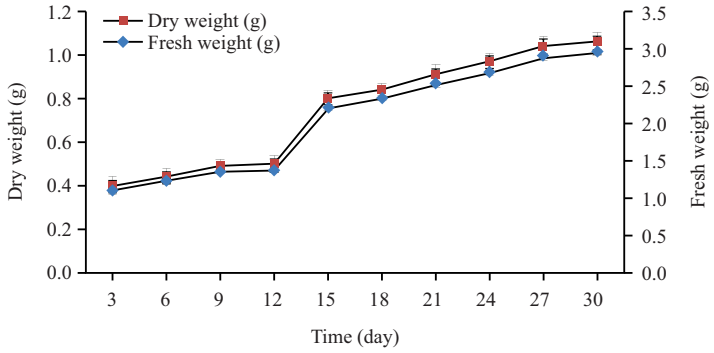

Fig. 3: Growth curve of *Astragalus fruticosus* callus on medium II
Mean±SD, n = 3

Fig. 4: Direct shoot induction in medium V

a sigmoidal shape with different growth phases. The medium composition can influence the duration of each phase[30]. The lag phase is characterized by weight accumulation, with no cell division[31]. In this study, the lag phase started from the beginning of the culture till the 9th day. The exponential phase showed maximum cell division and growth rates of calli, it occurred between the 9th and 15th days. The linear phase showed a reduction in cell division and an increase in cell volume[31]. It occurred from the 15th-24th day. Between the 24th and the 27th day, the deceleration phase was observed where, the calli must be transferred to a fresh medium, due to nutrients deprivation and toxic substances accumulation[30]. The stationary phase began on the 28th day when no cell division or weight increasing occurs. Growth parameters were as following:

GI	=	2.45	RGR	=	0.04
μ	=	0.03	dt	=	17.8 days

Shoot induction from *in vitro* germinated seedlings: The highest percentage of explants giving shoot (75.8%) was observed on Medium V, with about 10-25 shoot per explants, with length range (3-8 cm) after the third subculture as shown in Fig. 4. It was reported that BAP was the most commonly used cytokinin for *in vitro* culture of different *Astragalus* species[17].

In vitro* propagation of *Astragalus fruticosus

Regeneration of *Astragalus fruticosus* plantlet via somatic embryogenesis: Continuous subculture of calli on Medium I and Medium II, different forms of somatic embryos including globular, heart and torpedo-shaped forms were detected Fig. 5a-c. Upon subculture of embryogenic calli on hormonal free medium only calli browning was detected. Mature embryos on Medium II germinated successfully into cotyledonary embryos then into cotyledonary leaves of 0.5-1.0 cm height (Fig. 2b). Embroid on Medium I showed further development giving small plantlets of 1.3-2.4 cm height (Fig. 5d). Embryogenic callus induction was promoted by a high concentration of auxins as 2,4-D that causes hypermethylation of nuclear DNA resulting in the formation of embryogenic cells[32]. So, calli with high embryogenic potential were observed in medium II then medium I. Somatic embryogenesis was used for *in vitro* regeneration of species as *Astragalus melilotoides* using a combination of 2.69 μM NAA and 4.44 μM BAP[32].

Regeneration of *Astragalus fruticosus* plantlets via organogenesis: Upon culturing of shoots from Medium V into rooting media, roots started to appear after 4 weeks. The plantlets showed well-developed roots (0.5-3 cm in length)

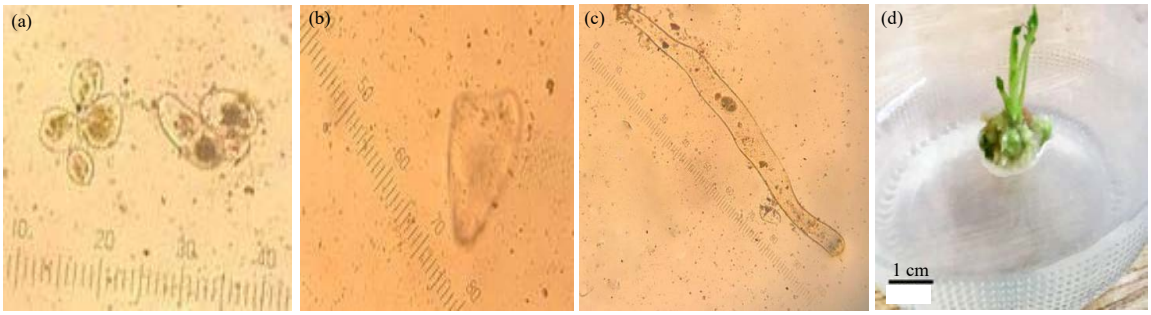

Fig. 5(a-d): Stages of embryogenic calli of *A. fruticosus* and plantlet regeneration via somatic embryogenesis, (a) Globular-shaped embryo, (b) Heart-shaped embryo, (c) Torpedo-shaped embryo and (d) Developed plantlet from a somatic embryo on medium I

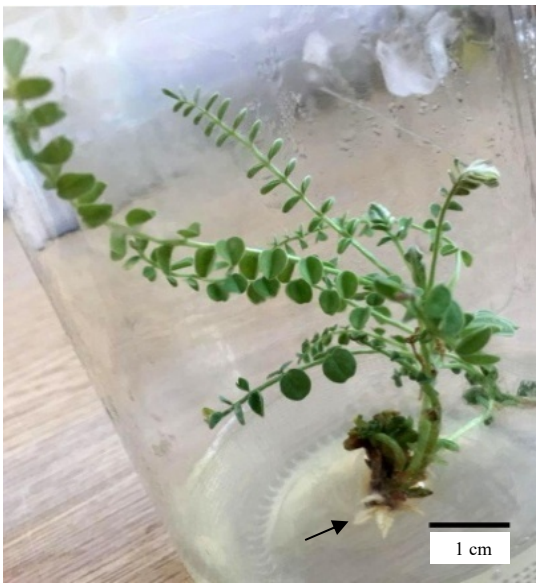

Fig. 6: Root induction in rooting medium

Fig. 7: Regenerated plant after transferring into the soil

3 weeks later as shown in Fig. 6. Smaller concentrations of auxins were more favourable for rhizogenesis than higher concentrations as higher auxins concentrations promoted callusing before roots induction resulting in an impaired vascular connection between the induced roots and shoot system. In the current research, a half-strength MS medium supplemented with 1 mg L^{-1} NAA was utilized for roots induction, in agreement with results reported for *Astragalus cicer in vitro* propagation system[33].

Acclimatization of the *in vitro* regenerated plantlets: Regenerated plantlets were transplanted into pots containing sterile soil: sand (1:1) as shown in Fig. 7. Pots were maintained in the greenhouse, plantlets can survive for two months in the soil after transplantation.

Results of biological activities

Cytotoxic activity: As shown in Table 3, the micropropagated plantlets ethanolic extract showed a stronger cytotoxic activity against MCF-7 cells, with $IC_{50} = 28.3 \pm 0.7$ µg mL^{-1} than its activity against HL-60 cells, with $IC_{50} = 49 \pm 1.9$ µg mL^{-1} compared to Cisplatin standard with $IC_{50} = 5.69 \pm 0.57$ and 7.74 ± 0.62 µg mL^{-1}, respectively. The reported results indicated that *Astragalus* saponins, polysaccharides and flavonoids have antitumor activities[34]. It was reported that there is a continuous increase in the global demand for anticancer drugs[35]. Hence, *Astragalus fruticosus* could be considered a precious drug to fight cancer.

Antidiabetic activity: The *in vitro* micropropagated plant ethanolic extract significantly inhibited the α-glucosidase enzyme. Percentages of α-glucosidase enzyme inhibition by

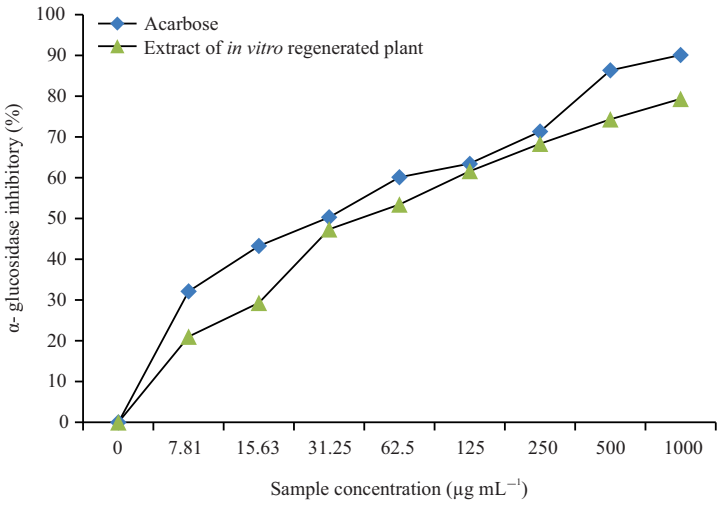

Fig. 8: α-Glucosidase inhibitory activity of the *in vitro* regenerated plantlets extract

Table 3: Cytotoxicity of plantlet extract against MCF-7 and HL-60 cell lines

		Extract concentration (µg mL^{-1})									
		0	3.9	7.8	15.6	31.25	62.5	125	250	500	
Cell line	Plant part used	Cell viability (%)									IC_{50}
MCF-7	Micropropagated	100	98.73	94.06	79.14	43.29	28.75	12.94	5.87	2.34	8.3
HL-60	Plantlet extract	100	100	98.59	88.24	64.27	39.16	24.52	13.96	6.23	49

Table 4: Total phenolic and flavonoid contents in the ethanolic extract of callus and *in vitro* cultured *Astragalus fruticosus*

Plant part used	Total phenolic (mg GAE g^{-1})	Total flavonoid (mg QE g^{-1})
Callus extract	8.73±0.54	15.38±0.64
In vitro plant extract	15.95±0.79	12.24±0.62

different concentrations of tested sample (0-1000) µg mL^{-1}, compared to acarbose standard were shown in Fig. 8. The IC_{50} value of the tested extract was 44.8±1.7 compared to the acarbose standard with IC_{50} 30.57±1.2 as shown in Fig. 9. Previous studies estimated the α-glucosidase inhibitory action of other *Astragalus* species specially *Astragalus membranaceus* root where the ethanolic extracts showed α-glucosidase inhibitory activity of 49.71 µg mL^{-1}. The α-glucosidase inhibitory activity was suggested to be mainly attributed to the presence of flavonoids and phenolic compounds[36].

Estimation of total phenolic and flavonoid contents: Total phenolics concentrations were estimated in the callus and *in vitro* plant ethanolic extracts using the Folin Ciocalteu method. The results as shown in Table 4 were estimated in terms of Gallic Acid Equivalent (GAE) from the standard curve. The phenolics concentrations in the callus and *in vitro* plant extracts were 8.73±0.54 and 15.95±0.79 mg g^{-1}, respectively.

Total flavonoids contents of the callus and *in vitro* plant extracts were evaluated by the aluminium chloride method in terms of Quercetin Equivalent (QE)as shown in Table 4 and were deduced from the standard curve. Flavonoid concentrations in the callus and *in vitro* plant extracts were 15.38±0.64 and 12.24±0.62 mg g^{-1}, respectively. In this research, the total phenolic and flavonoid contents in addition to their compositions in the extracts of callus and *in vitro* cultured plants were examined for the first time. It was reported that phenolic and flavonoid contents of the flowering aerial parts of the wild *A. squarrosus* were 23.3 and 26.0 mg g^{-1}, respectively[11] that slightly higher than our results. The higher values of phenolic and flavonoid contents of the wild plants may be attributed to their exposure to different stress circumstances in the field environment that caused a raising in the phenolic constituents for adaptation and survival. Whereas, the *in vitro* grown plants had no stress conditions and so, no need for over-production of phenolics[37].

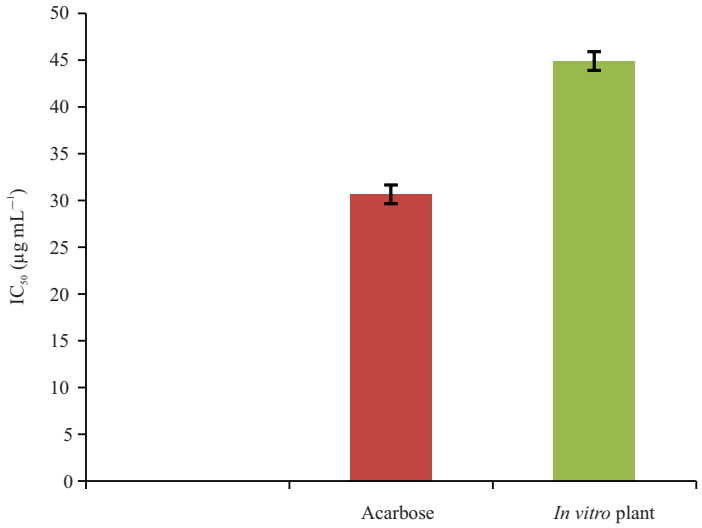

Fig. 9: Antidiabetic IC_{50} (µg mL^{-1}) of the *in vitro* regenerated plantlets extract

Table 5: HPLC analysis of phenolics and flavonoids in the ethanolic extract of callus and *in vitro* cultured *Astragalus fruticosus*

	Concentration of compounds (mg mL^{-1})	
Identified compounds	*In vitro* plant	Callus
Pyrogallol	14.12	-
Eugenol	-	-
Caffeic acid	-	6.51
Gallic acid	-	7.14
Protocatechuic acid	20.41	8.12
p-Coumaric acid	7.26	-
Syringic acid	-	8.10
Chlorogenic acid	5.06	-
Ferulic acid	8.31	-
Ellagic acid	5.66	15.36
Ascorbic acid	-	-
Rutin	-	-
Naringin	-	-
Apigenin	-	10.05
Luteolin	8.09	22.41
Kaempferol	13.30	-
3-Hydroxyflavone	-	9.48
Myricetin	-	-

Analysis of extracts by High-Performance Liquid Chromatography (HPLC): Phenolic and flavonoid compositions of the tested extracts were examined by HPLC as shown in Table 5. Protocatechuic, ellagic acid and luteolin were identified in both extracts. While, pyrogallol, *p*-coumaric acid, chlorogenic acid and kaempferol were identified only in the *in vitro* plant extracts. Caffeic, gallic and syringic acids were identified only in the callus extracts. Protocatechuic acid is the main phenolic compound in the *in vitro* plant extracts (20.41 mg mL^{-1}) while, ellagic acid is the main phenolic compound in the callus extracts (15.36 mg mL^{-1}). Luteolin (22.41 mg mL^{-1}) and kaempferol (13.30 mg mL^{-1}) are the main flavonoids in the extracts from the callus and the *in vitro* plants, respectively.

CONCLUSION

In the presented study, plant micropropagation of *A. fruticosus* has been achieved. Somatic embryogenesis as well as direct organogenesis are efficient regeneration systems that might be suitable for further biotechnological approaches. To the best of our knowledge, there is no report for *Astragalus fruticosus* micropropagation. Moreover, the micropropagated plants showed promising cytotoxic and

antidiabetic activities, in addition to reasonable phenolic and flavonoid contents that all screened for the first time.

SIGNIFICANCE STATEMENT

This study revealed the best condition for seed germination, callus and shoot induction to develop an efficient micropropagation protocol for the endangered *Astragalus fruticosus* Forssk. plant. Moreover, the study will help the researchers to perform further biotechnological studies on this plant and other related plant species. Also, our results suggested that *Astragalus fruticosus* could serve as a precious source of bioactive agents to fight global health problems like cancer and diabetes.

REFERENCES

1. El-Ghani, M.M.A., A.S.A. El-Sayed, A. Moubarak, R. Rashad, H. Nosier and A. Khattab, 2021. Biosystematic study on some Egyptian species of *Astragalus* L. (fabaceae). Agriculture, Vol. 11. 10.3390/agriculture11020125.
2. Li, X., L. Qu, Y. Dong, L. Han and E. Liu *et al*., 2014. A review of recent research progress on the *Astragalus* genus. Molecules, 19: 18850-18880.
3. Allam, R.M., D.A. Selim, A.I. Ghoneim, M.M. Radwan and S.M. Nofal *et al*., 2013. Hepatoprotective effects of *Astragalus kahiricus* root extract against ethanol-induced liver apoptosis in rats. Chin. J. Nat. Med., 11: 354-361.
4. Radwan, M.M., N.A. El-Sebakhy, A.M. Asaad, S.M. Toaima and D.G.I. Kingston, 2007. Spinocoumarin I, a new coumarin derivative from *Astragalus spinosus* Forssk. Nat. Prod. Commun., Vol. 2. 10.1177/1934578x0700200910.
5. Ionkova, I., A. Shkondrov, I. Krasteva and T. Ionkov, 2014. Recent progress in phytochemistry, pharmacology and biotechnology of *Astragalus* saponins. Phytochem. Rev., 13: 343-374.
6. Abbas, F. and R. Zayed, 2005. Bioactive saponins from *Astragalus suberi* L. growing in Yemen. Zeitschrift für Naturforschung C, 60: 813-820.
7. Gorai, D., S.K. Jash and R. Roy, 2016. Flavonoids from *Astragalus* genus. Int. J. Pharm. Sci. Res., 7: 2732-2747.
8. Xia, Y.G., S.M. Yu, J. Liang, B.Y. Yang and H.X. Kuang, 2020. Chemical fingerprinting techniques for the differentiation of polysaccharides from genus *Astragalus*. J. Pharm. Biomed. Anal., Vol. 178. 10.1016/j.jpba.2019.112898.
9. Block, K.I. and M.N. Mead, 2003. Immune system effects of echinacea, ginseng and astragalus: A review. Integr. Cancer. Ther., 2: 247-267.
10. Cho, W.C.S. and K.N. Leung, 2007. *In vitro* and *in vivo* anti-tumor effects of *Astragalus* membranaceus. Cancer Lett., 252: 43-54.
11. Asgarpanah, J., S.M. Motamed, A. Farzaneh, B. Ghanizadeh and S. Tomraee, 2011. Antioxidant activity and total phenolic and flavonoid content of *Astragalus squarrosus* Bunge. Afr. J. Biotech., 10: 19176-19180.
12. Zayed, R. and M. Wink, 2004. Induction of tropane alkaloid formation in transformed root cultures of brugmansia suaveolens (*Solanaceae*). Zeitschrift für Naturforschung C, 59: 863-867.
13. Zayed, R., M. Wink and H. El-Shamy, 2006. *In vitro* organogenesis and alkaloid accumulation in datura innoxia. Zeitschrift für Naturforschung C, 61: 560-564.
14. Zayed, R. and M. Wink, 2009. Induction of pyridine alkaloid formation in transformed root cultures of *Nicotiana tabacum*. Zeitschrift für Naturforschung C, 64: 869-874.
15. Zayed, R., H. El-Shamy, S. Berkov, J. Bastida and C. Codina, 2011. *In vitro* micropropagation and alkaloids of *Hippeastrum vittatum. In Vitro* Cell. Dev. Biol. Plant, 47: 695-701.
16. Zayed, R., 2011. Efficient *in vitro* elicitation of β-carboline alkaloids in transformed root cultures of *Peganum harmala*. Bull. Fac. Pharm. Cairo Univ., 49: 7-11.
17. Hasancebi, S., N.T. Kara, O. Cakir and S. Ari, 2011. Micropropagation and root culture of Turkish endemic *Astragalus chrysochlorus* (Leguminosae). Turk. J. Bot., 35: 203-210.
18. Albayrak, S. and O. Kaya, 2019. Antioxidant, antimicrobial and cytotoxic activities of endemic *Astragalus argaeus* Boiss. from Turkey. Hacettepe J. Biol. and Chem., 47: 87-97.
19. Godoy-Hernández, G. and F.A. Vázquez-Flota, 2012. Growth Measurements: Estimation of Cell Division and Cell expansion, In: Plant Cell Culture Protocols. Loyola-Vargas V.M. and N. Ochoa-Alejo (Eds.)., Humana Press, Totowa, New Jersey, ISBN: 978-1-61779-818-4 pp: 41-48.
20. Parsaeimehr, A., E. Sargsyan and K. Javidnia, 2010. A comparative study of the antibacterial, antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of *Ephedra*. Molecules, 15: 1668-1678.
21. Kebeish, R., A. El-Sayed, H. Fahmy and A. Abdel-Ghany, 2016. Molecular cloning, biochemical characterization and antitumor properties of a novel L-asparaginase from *Synechococcus elongatus* PCC6803. Biochem. (Moscow), 81: 1173-1181.
22. Gomha, S.M., S.M. Riyadh, E.A. Mahmmoud and M.M. Elaasser, 2015. Synthesis and anticancer activities of thiazoles, 1,3-thiazines and thiazolidine using chitosan-grafted-poly (vinylpyridine) as basic catalyst. Heterocycles, 91: 1227-1243.
23. Shai, L., S. Magano, S. Lebelo and A. Mogale, 2011. Inhibitory effects of five medicinal plants on rat alpha-glucosidase: Comparison with their effects on yeast alpha-glucosidase. J. Med. Plant Res., 5: 2863-2867.

24. Mohammed, H.S., M.M. Abdel-Aziz, M.S. Abu-Baker, A.M. Saad, M.A. Mohamed and M.A. Ghareeb, 2019. Antibacterial and potential antidiabetic activities of flavone C-glycosides isolated from *Beta vulgaris* subspecies cicla L. var. flavescens (Amaranthaceae) cultivated in Egypt. Curr. Pharm. Biotechnol., 20: 595-604.
25. Meda, A., C.E. Lamien, M. Romito, J. Millogo and O.G. Nacoulma, 2005. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey as well as their radical scavenging activity. Food Chem., 91: 571-577.
26. Siddique, N.A., M. Mujeeb, A.K. Najmi and M. Akram, 2010. Evaluation of antioxidant activity, quantitative estimation of phenols and flavonoids in different parts of *Aegle marmelos*. Afr. J. Plant Sci., 4: 1-5.
27. Zuo, Y., H. Chen and Y. Deng, 2002. Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta, 57: 307-316.
28. Schnadelbach, A., L. Veiga-Barbosa, C. Ruiz and F. Pérez-García, 2016. Dormancy breaking and germination of *Adenocarpus desertorum*, *Astragalus gines-lopezii* and *Hippocrepis grosii* (Fabaceae) seeds, three threatened endemic spanish species. Seed Sci. Technol., 44: 1-14.
29. Erisen, S., M. Yorgancilar, E. Atalay and M. Babaoglu, 2010. Prolific shoot regeneration of *Astragalus cariensis* boiss. Plant Cell, Tissue Org. Cult. (PCTOC), 100: 229-233.
30. Smith, R.H., 2012. Plant Tissue Culture: Techniques and Experiments. Academic Press, San Diego, pp: 71.
31. Dos Santos, M.R.A., M. das Gracas Rodrigues Ferreira and V. Sarubo, 2010. Determination of callus growth curve in conilon coffee. Rev. Caatinga, 23: 133-136.
32. Hou, S.W. and J.F. Jia, 2004. High frequency plant regeneration from *Astragalus melilotoides* hypocotyl and stem explants via somatic embryogenesis and organogenesis. Plant Cell, Tissue Org. Cult., 79: 95-100.
33. Basalma, D, S. Uranbey, D. Gürlek and S. Özcan, 2008. TDZ-induced plant regeneration in *Astragalus cicer* L. Afr. J. Biotechnol., 7: 955-959.
34. Ionkova, I., 2008. Anticancer compounds from *in vitro* cultures of rare medicinal plants. Pharmacogn. Rev., 2: 206-218.
35. El-Sayed, A.S.A., N.Z. Mohamed, S. Safan, M.A. Yassin and L. Shaban *et al*., 2019. Restoring the taxol biosynthetic machinery of *Aspergillus terreus* by *Podocarpus gracilior* pilger microbiome, with retrieving the ribosome biogenesis proteins of WD40 superfamily. Sci. Rep., Vol. 9. 10.1038/s41598-019-47816-y.
36. Janibekov, A.A., F.S. Youssef, M.L. Ashour and N.Z. Mamadalieva, 2018. New flavonoid glycosides from two *Astragalus* species (Fabaceae) and validation of their antihyperglycaemic activity using molecular modelling and *in vitro* studies. Ind. Crops Prod., 118: 142-148.
37. Yildirim, A., E. Uyar and A. Turker, 2020. *In vitro* culture of endemic *Astragalus gymnolobus* fischer and comparison of its antibacterial, antioxidant and phenolic profiles with field grown plants. J. Agri. Sci. Technol., 22: 815-828.