MULTIPLE SHOOT REGENERATION OF *Ceratophyllum demersum* L. ON AGAR SOLIDIFIED AND LIQUID MEDIUMS

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ABSTRACT

Coontail or hornwort (*Ceratophyllum demersum* L.) is a submergent aquatic macrophyte from Ceratophyllaceae family that has been widely used to remove heavy metals (photoremediation) and for pollution monitoring (biomonitor) in the aquatic environment. Besides that, it is used as aquatic plant and as a source of food for some livestock and poultry. Shoot tips, 1st and 2nd nodal explants of *C. demersum* were cultured on agar solidified or liquid MS medium supplemented with 0.05-0.80 mg/L BA. Multiple shoot regeneration without callus induction was recorded on both agar solidified and liquid culture media. However, earlier shoot induction and more mean number of shoots per explant with longer shoots were recorded on liquid culture medium compared to solidified medium. Maximum shoot regeneration frequency from all explants on both culture mediums was obtained at 0.05 mg/L BA. Comparing explant types, 2nd nodal meristem explants gave more number of shoots per explants (16.75 and 204.33 from solid and liquid culture medium), respectively. *In vitro* regenerated plantlets were successfully acclimatized in aquariums, and plants can be easily acclimatized at slight acidic to slight alkaline pH levels.

KEYWORDS:
Agar solidified, aquatic, liquid, shoot regeneration

1. INTRODUCTION

Aquatic plants are the natural elements of water environment that produce oxygen and organic matters, and they supply protection, feeding and breeding environment for other aquatic organisms [1]. Some aquatic plants are used for the purpose of phytoremediation because they remove nutrients and heavy metals from water environment [2-4]. Additionally, they are used for monitoring pollution (biomonitor) in the aquatic environment [5, 6].

Coontail or hornwort (*Ceratophyllum demersum* L.) is a submergent aquatic macrophyte belonging to Ceratophyllaceae family [7, 8]. The plant has been widely used to remove heavy metals (photoremediation) [9-12], and for pollution monitoring (biomonitor) [13, 14] in the aquatic environment. *C. demersum* is also one of the popular plants in aquatic industry [15, 16] due to tolerating a wide range of aquatic conditions [17]. It also provides an excellent living environment for shelter to fish and aquatic organisms [7, 18]. It is also used as a source of food for some livestock, poultry [19], and fish [20].

C. demersum is a traditional medicine plant that has been used in the treatment of ulcer, diarrhoea, dysentery, wounds, fever, burning sensation, haemorrhoids or piles, intrinsic haemorrhages, hyperdipsia, epistaxis and haematemesis. Additionally, glycosides, flavonoids, alkaloids, steroids and tannins have been isolated from the methanolic and aqueous extracts of *C. demersum* [21]. Furthermore, other important chemical compounds, such as tricin-7-O-β-D-glucoside, naringenin-7-O-β-D-glucoside, esculetin, β-sitosterol, 7α-hydroxyl-β-sitosterol, 7α-methoxyl-β-sitosterol and palmitic acid have also been isolated from *C. demersum* [22].

There is no report available on *in vitro* propagation of *C. demersum*, an important medicinal aquatic plant that can also be used for photoremediation and biomonitoring of water pollution.

2. MATERIALS AND METHODS

The plants of *C. demersum* were obtained from a local aquarium of Karaman province in Turkey. After taxonomic studies, 3-5 cm long twigs containing 5-6 nodes with attached leaves were washed under tap water for 15 min. Surface sterilization was performed with 10 % H_2O_2 for 7 min, followed by rinsing thrice with sterilized distilled water, under continuous stirring for 5 min each. After sterilization, leaves were detached from twigs and shoot meristem, 1st and 2nd nodal meristem explants were isolated under sterile conditions and cultured on MS [23] medium devoid of growth variants for 2 weeks to obtain contamination-free explants. Thereafter, explants were cultured on MS medium supplemented with 3% sucrose and 0.05, 0.10, 0.20, 0.40 and 0.80 mg/L BA (Table 1) in Magenta GA7 vessels solidified with 0.65% agar or without agar (liquid culture).

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The experiment was run in triplicate with the pH of all media adjusted to 5.8±1 before autoclaving (118 kPa atmospheric pressure, 120 °C for 21 min). All cultures were incubated under 16-h light photoperiod (1500 lux) using white LEDs (Light Emitting Diodes) lights. The data for both experiments (agar solidified and liquid culture) were recorded for 8 weeks of culturing.

The regenerated shoots were acclimatized in jars containing water at variable pHs of 4.0-10.0 [24], and then left open for acclimatization in a growth room at 23 °C with 16-h light photoperiod for 30 days. Thereafter, plants were transferred to aquariums for further acclimatization.

Each treatment contained 8 explants and was replicated 6 times (8 x 6 = 48 explants), and repeated twice. Statistical analysis was performed as one-way ANOVA using SPSS17 for Windows, and post hoc tests were performed using Duncan`s test. Data given in percentages were subjected to arcsine transformation [25] before statistical analysis.

3. RESULTS

Shoot meristem, 1st and 2nd nodal meristem explants were cultured on agar solidified and liquid culture mediums containing different concentrations of N 6-benzyladenine (BA; 0.05-0.80 mg/L). All explants were also cultured on MS medium without BA as control experiment. No callus formation was recorded on all explants in both solidified and liquid culture medium (Figs. 1 a, b, and c). Shoot regeneration in agar solidified medium was observed after 2 weeks of culture, followed by multiple shoot regeneration after 4 weeks of culture. On the other hand, shoot induction from liquid started within one week and after two weeks of culture, multiple shoot regeneration was recorded. Comparing explants, shoot induction from shoot meristem explant was earlier compared to other nodal meristems, irrespective of culture medium. Data regarding frequency of shoot regeneration (%), mean number of shoots per explants and mean shoot length of both experiments were recorded after 8 weeks of culturing (Figs. 1 d, e, and f).

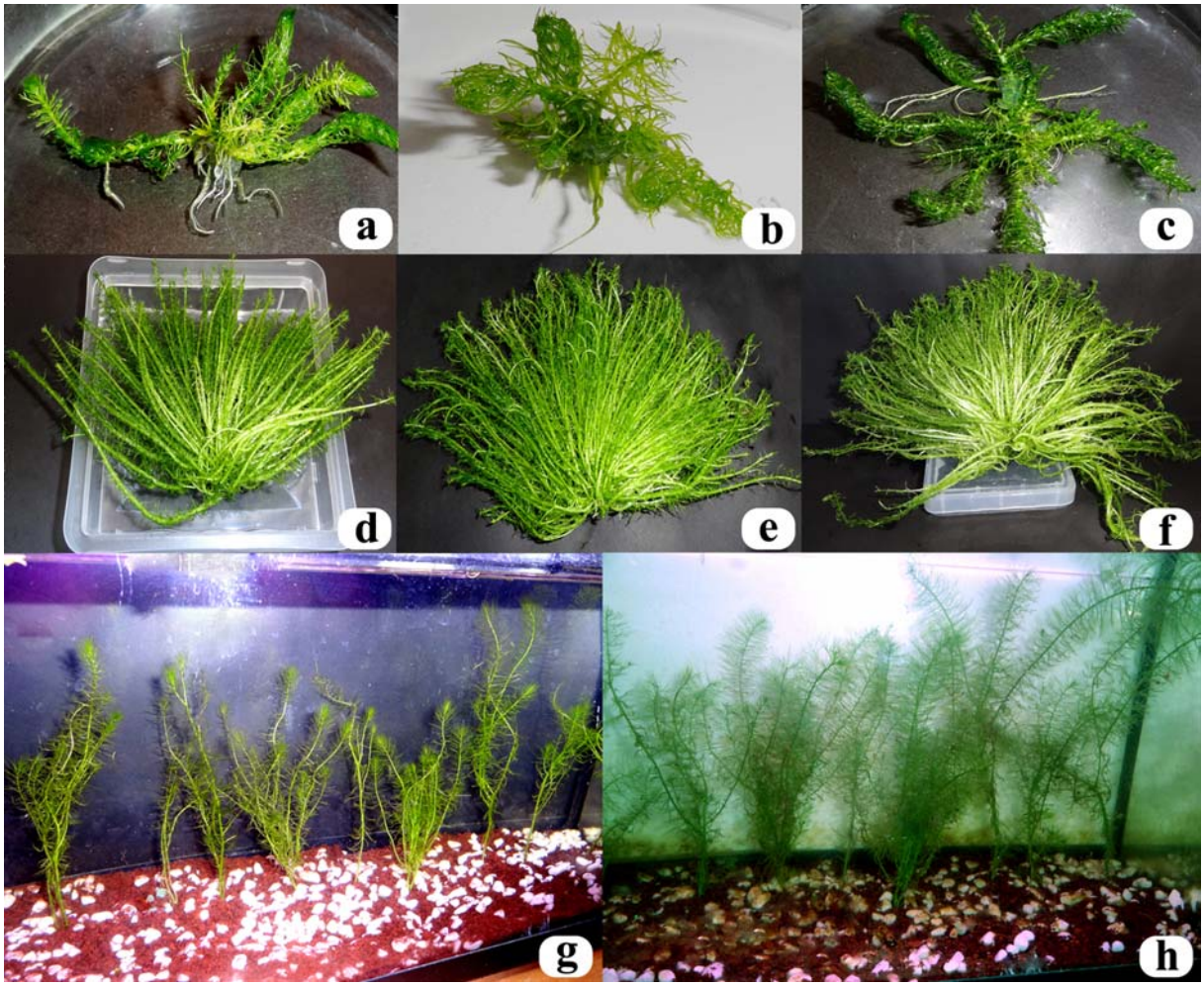

FIGURE 1 - *In vitro* shoot regeneration and acclimatization of *C. demersum:* (a) shoot tip, (b) 1st, (c) 2nd nodal segment, after 4 weeks of culturing, and (d) shoot tip, (e) 1st, (f) 2nd nodal segment after 8 weeks of culturing, acclimatized plant in aquariums after (g) 4 weeks and (h) 8 weeks.

Shoot regeneration frequency on agar-solidified medium was found statistically insignificant, and was recorded to be 91.67-100.00 % from shoot meristem explants and 75.00-100.00 % from both 1st and 2nd nodal meristem explants. 100 % shoot regeneration frequency from all explants was observed on MS medium supplemented with 0.05 mg/L BA whereas, on MS medium devoid of growth variants, it was recorded to be 91.67 % for shoot tips and 1st nodal segments but 75.00 % for 2nd nodal segments (Table 1). On the other hand, statistically significant effects of BA concentrations were observed on shoot regeneration frequency of liquid culture medium. Shoot regeneration frequency of shoot meristem, 1st and 2nd nodal meristem explants ranged between 65-100%, 60-90% and 25-90%, respectively on MS liquid medium. Maximum shoot regeneration frequency for all explants was recorded on MS medium containing 0.05 mg/L BA. In general, further increase in BA concentration resulted in decreased shoot regeneration frequency for all explants. However, the decline in shoot regeneration was more prominent on 2nd nodal meristem explants compared to other explants used in the experiment. Comparing shoot regeneration frequency of explants, shoot meristem had more shoot regeneration compared to other explants on all culture mediums. Shoot regeneration frequency on MS medium was recorded to be 83.33-91.67 % and 55.00-100.00 % on agar-solidified culture medium and liquid culture medium, respectively (Table 1).

Mean numbers of shoots per explant from both culture media were found to be statistically significant. Relatively more numbers of shoots per explants were recorded on liquid culture medium compared to agar-solidified medium. Mean number of shoots per explant obtained from agar-solidified medium ranged between 2.66-11.41, 4.92-11.95 and 5.75-16.75 from shoot meristem, 1st and 2nd nodal meristem explants, respectively, and 1.36-1.75 per explant on MS-solidified medium. Maximum numbers of shoots per explant were scored on 0.80 mg/L BA whereas minimum numbers of shoots per explant were recorded on 0.05 mg/L BA-containing medium (Table 2). Shoots per explants increased with increase in BA concentrations, irrespective of explant type. Comparing explant type, 2nd nodal meristem explants generated more numbers of shoots per explants compared to shoot meristem and 1st nodal meristem, irrespective of BA concentration. Liquid culture medium also showed the similar response to mean numbers of shoots per explants. However, average number of shoots per explant was relatively too high compared to agar-solidified medium. Mean numbers of shoots per explant were recorded to be 40.87-169.07, 47.55-109.43 and 52.88-204.33 from shoot meristem, 1st and 2nd nodal meristem explants, respectively (Table 2). 1.50-2.20 shoots per explant were recorded from MS liquid medium. Likewise agar-solidified medium, mean number of shoots per explant also decreased with increase in BA concentration. Similarly, irrespective of BA concentration in the culture medium, 2nd nodal meristem explants gave more numbers of shoots per explants compared to shoot meristem and 1st nodal meristem.

Data on mean shoot length showed that relatively longer shoots were obtained from MS liquid medium compared to MS media containing BA concentrations on solidified culture medium. Mean shoot length of shoot meristem, 1st and 2nd nodal meristem explants were recorded as 0.43-1.94 cm, 0.38-1.97 cm and 0.42-2.44 cm, respectively (Table 3). Increase of BA concentration re-

TABLE 1 - Shoot regeneration frequency of *C. demersum* from agar solidified medium and liquid medium containing BA.

BA (mg/L)	Agar solidified culture medium			Liquid culture medium		
	Shoot tip	1st nodal meristem	2nd nodal meristem	Shoot tip	1st nodal meristem	2nd nodal meristem
0.05	100.00^{ns}	100.00^{ns}	100.00^{ns}	100.00^{a}	90.00^{a}	90.00^{a}
0.10	100.00	100.00	91.67	90.00^{a}	75.00^{abc}	75.00^{ab}
0.20	100.00	100.00	100.00	65.00^{b}	80.00^{ab}	65.00^{abc}
0.40	91.67	100.00	100.00	90.00^{a}	60.00^{c}	40.00^{bc}
0.80	91.67	75.00	75.00	80.00^{ab}	70.00^{bc}	25.00^{c}
MSO	91.67	91.67	83.33	100.00^{a}	75.00^{abc}	55.00^{abc}

Means followed by different small letters within columns are significantly different using Duncan *p<0.01*.

TABLE 2 - Mean number of shoots per explant of *C. demersum* from agar solidified medium and liquid medium containing BA.

BA (mg/L)	Agar solidified culture medium			Liquid culture medium		
	Shoot tip	1st nodal meristem	2nd nodal meristem	Shoot tip	1st nodal meristem	2nd nodal meristem
0.05	2.66^{bc}	4.92^{bc}	5.75^{cd}	40.87^{d}	47.55^{b}	52.88^{e}
0.10	5.00^{abc}	6.08^{b}	6.61^{c}	89.16^{c}	95.13^{a}	85.25^{d}
0.20	8.50^{ab}	11.17^{a}	11.47^{b}	143.97^{b}	102.67^{a}	146.50^{c}
0.40	9.44^{a}	11.50^{a}	12.25^{ab}	157.43^{ab}	106.47^{a}	204.33^{a}
0.80	11.41^{a}	11.95^{a}	16.75^{a}	169.07^{a}	109.43^{a}	170.11^{b}
MSO	1.36^{c}	1.58^{c}	1.75^{e}	1.50^{e}	2.17^{c}	2.20^{f}

Means followed by different small letters within columns are significantly different using Duncan *p<0.01*.

TABLE 3 - Mean shoot length of *C. demersum* from agar solidified medium and liquid medium containing BA.

BA (mg/L)	Agar solidified culture medium			Liquid culture medium		
	Shoot tip	1st nodal meristem	2nd nodal meristem	Shoot tip	1st nodal meristem	2nd nodal meristem
0.05	1.94^{b}	1.97^{b}	2.44^{ab}	2.91^{c}	2.89^{d}	2.96^{cd}
0.10	1.38^{b}	1.78^{b}	2.28^{bc}	3.56^{c}	5.02^{ab}	4.02^{bc}
0.20	1.35^{b}	0.90^{bc}	1.75^{c}	5.72^{a}	5.65^{a}	6.38^{a}
0.40	0.44^{c}	0.62^{c}	0.52^{d}	3.97^{bc}	3.98^{c}	4.52^{b}
0.80	0.43^{c}	0.38^{c}	0.42^{d}	3.08^{c}	2.18^{d}	1.77^{d}
MSO	3.34^{a}	4.00^{a}	2.97^{a}	5.26^{ab}	4.33^{bc}	3.15^{bcd}

Means followed by different small letters within columns are significantly different using Duncan $p<0.01$.

sulted in decreased shoot length, and BA concentrations >0.40 mg/L resulted in stunted shoots. Maximum shoot length was recorded on MS medium supplemented with 0.05 mg/L BA. Contrarily, liquid culture exerted different response to mean shoot length of all explants compared to solidified medium. Relatively longer shoots were recorded from all explants, irrespective of BA concentrations. Maximum shoot lengths from all explants were scored on MS medium supplemented with 0.20 mg/L BA, followed by decreased shoot length. However, these shoots were enough longer with minimum shoot length (1.77 cm) was obtained from 2nd nodal meristem explant. Mean shoot lengths ranged between 2.91-5.72 cm, 2.18-5.65 cm and 1.77-6.38 cm, recorded from shoot meristem, 1st and 2nd nodal meristem explants, respectively (Table 3).

C. demersum is a non-rooted plant in natural conditions. However, rhizoid formation was also recorded on regenerated shoots both in agar-solidified and liquid mediums at variable frequency, irrespective of explant type (data not shown). Rhizoid formation was prominent on shoots obtained from MS medium without BA in both solidified and liquid culture media. Rhizoid induction on liquid culture medium was recorded only on MS medium supplemented with 0-0.40 mg/L BA. Contrarily, 1st and 2nd nodal meristem explants induced rhizoids at variable frequency on all culture media (data not shown). For acclimatization, *in vitro* regenerated shoots with and without rhizoids were transferred to jars containing water with pH values of 4.0-10.0, in order to find out the best pH for plant growth and development in aquariums. Results showed that plants did not gain length at pH 4.0, followed by pH 5.0. On the other hand, pH 10.0 also resulted in less shoot growth whereas pH 7.0 was found optimum for shoot growth, followed by pH values of 8.0 and 9.0. Thereafter, shoots with or without rhizoids were successfully transferred to aquariums (Fig. 1 g), and 100 % survival rate was recorded after 8 weeks (Fig. 1 h).

4. DISCUSSION

The study presents the efficient multiple shoot regeneration of the important medicinal plant *C. demersum* cultured on 0.05-0.80 mg/L BA containing media. Multiple shoot regeneration from shoot meristem and nodal meristems has been reported in other aquatic plants like *Mentha viridis* [26], *Stevia rebaudiana* [27], *Vitex negundo* [28], and *Marsdenia brunoniana* [29]. Experiments were performed in two different media, agar-solidified and liquid culture medium. Results showed the clear effects of culture media on shoot regeneration behavior as early regeneration was recorded on liquid culture medium compared to solidified medium. This might be due to the active uptake of plant hormones by *C. demersum* which like to live in aquatic conditions. Contrarily, Şumlu [30] reported death of shoot meristem, 1st and 2nd nodal meristem explants, 1st and 2nd internodal segments of *Rotala macrandra* on MS liquid medium containing 0.25 mg/L BAP -0.50 mg/L NAA and 0.50 mg/L BAP – 0.50 mg/L NAA, after 4 weeks of culturing.

Results further showed that BA concentrations failed to induce callus induction and led to organogenesis in both culture mediums. Contrarily, callus induction was reported at basal ends of nodal segments cultured on different concentrations of BA, kinetin and 2-iP [31], internodal and leaf explants of *B. monnieri* cultured on various concentrations of BA-NAA [24]. The difference in the results might be due to the presence of NAA in the culture medium, and difference of plant and explant type used in the experiments. Results on shoot regeneration frequency showed that agar-solidified medium was more responsive to BA concentration than liquid culture medium, irrespective of explant type in line with the findings of Shahzad *et al*. [32] in *Veronica anagallis-aquatica,* who also reported more shoot regeneration frequency from agar-solidified media with regard to liquid ones. Contrarily, liquid culture was reported to be more better than agar-solidified medium for shoot regeneration frequency of *Nothapodytes mimmonia* [31], *Alocasia amazonica* [33] and *Bacopa monnieri* [34].

Results also showed that shoot meristem explant was more responsive than other explants, possibly due to age and presence of more numbers of young and active dividing cells in the meristem, in line with [35]. Maximum shoot regeneration at low concentration of BA for all explants showed the efficiency of explants to BA concentrations. However, increased BA concentration had reducing effects on shoot regeneration frequency of all explants according to findings of Hassan and Roy [36], who also reported decreased shoot regeneration frequency from shoot apex and nodal segments of *Gloriosa superba* L. cultured at 2.0 and 2.5 mg/L BA. Similarly, Dandin and

Murthy [31] achieved maximum shoot regeneration (83 %) on 1-µM BA-containing medium whereas Gnanaraj *et al.* [37] reported increased shoot regeneration frequency with increased BA concentration in *Alternanthera sessili.*

Results further showed that the negative effect was specific to explant type, and 2nd nodal explants were affected more compared to other explants. Shoot meristem explants were more efficient than all other explants on all culture media. Öztürk [38] cultured shoot meristem, 1st nodal segment, leaf and petiol explants of *Hygrophila difformis* on TDZ-NAA-supplemented medium and obtained a maximum number of 52.63 shoots per explant, and more longer shoots (5.63 cm) from 1st nodal segments.

Results on mean number of shoots showed that liquid culture medium was far better (10 to 20-fold more shoots) than agar-solidified medium. Dandin and Murthy [31] cultured nodal explants of *N. nimmoniana* on liquid MS and semi-solid MS medium supplemented with 1, 2, 5 and 10 µM BAP, Kin and 2-iP, and got a maximum of 165.9 shoots per explant from liquid MS medium containing 2 µM BAP. Results further illustrated that mean number of shoots per explant increased with increase in BA concentrations, in line with Jo *et al.* [33] in *Alocasia amazonica*. Contrarily, Sharma *et al.* [39] reported negative effects of increased BA concentration on shoots per explant of *B. monnieri.* Gnanaraj *et al.* [37] reported a maximum number of shoots from shoot meristem explants of *A. sessilis* on MS medium containing 0.20 mg/L BA. Karatas *et al.* [24] also obtained maximum number of shoots at 0.25 mg/L BA-0.25 mg/l NAA from internodal segments and leaf explant of *B. monnieri*. Results further illustrated that all explants required relatively higher concentrations of BA for more shoot regeneration, irrespective of explant and culture medium type, according to results of Jo *et a.,* [33].

Results on mean shoot length also showed that agar-solidified medium hindered the shoot length compared to liquid medium, irrespective of explant type and BA concentrations. Increase of BA also caused the regeneration of stunted shoots which was more obvious at BA concentrations >0.40 mg/L on agar-solidified medium, contradictory to Vijaykumar *et al*. [40], who reported an increase in shoot length with an increase in BA and TDZ concentration in the culture medium using the leaf explant of *B. monnieri*. On the other hand, longer shoots were recorded on liquid medium with an optimum of 0.20 mg/L BA-containing medium. Hung *et al*. [41] cultured shoot meristems of *Wasabia japonica* on liquid MS, ½ MS and ¼ MS containing 0.5 µM BAP, and observed maximum longer shoots of 34.7±0.8 cm from MS medium, followed by 27.0±0.8 cm from ½ MS medium.

Irrespective of non-rooted plants in natural aquatic conditions, rhizoid formations on regenerated shoots supposed to be due to the presence of micro and macro elements, BA concentration, or explant type. Results further showed that rhizoid formation was specific to explant type and culture media. Acclimatization results showed that plants can be successfully acclimatized at natural to slightly alkaline pH levels. Highly acidic or alkaline pH levels hindered the plant growth, as also observed by Karatas *et al.* [24], who reported limited shoot length of *B. monnieri* at pH 4.0 and 10.0. Successful acclimatization of both shoots with or without rhizoids in aquariums showed the ability of regenerated plants to be transferred directly to aquariums from lab conditions. Similarly, successful acclimatization of *in vitro* regenerated aquatic plants had been reported for *R. macrandra* [30], *Veronica anagallis-aquatica* [32], *A. sessilis* [37], *Nymphoides indica* [42], *Cryptocoryne wendtii* and *Cryptocoryne beckettii* [43], *B. monnieri* [44] and *Hygrophila polysperma*[45]

Establishment of *in vitro* regeneration and acclimatization protocol of *C. demersum* provides an alternative way of propagation which can be applied to different biotechnological tools. This protocol also provides the availability of plants to be used for photoremediation and as bio-indicator, along with the extraction of medicinally important compounds from this important aquatic plant.

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