Vegetos (2019) 32:247–253
https://doi.org/10.1007/s42535-019-00042-3

RESEARCH ARTICLES

Evaluation of isoflavonoid content in context to tuber size and seed biology study of *Pueraria tuberosa* (Roxb.ex.Willd.) DC: a vulnerable medicinal plant

Bhanupriya Kanthaliya[1] · Abhishek Joshi[1] · Jaya Arora[1]

Received: 26 April 2019 / Revised: 9 July 2019 / Accepted: 13 July 2019 / Published online: 22 July 2019
© Society for Plant Research 2019

Abstract
Pueraria tuberosa known as "Kudzu" is a vulnerable perennial woody liana and native to South–East Asia. The tuber of this plant is used in traditional medicine system and possesses various pharmacological properties due to presence of various Isoflavonoids. The present study has been aimed to create GIS map of *P. tuberosa* with special reference to South Rajasthan. Analysis of total isoflavonoids content in tuber and its correlation with size and geographical distribution has also been done. HPLC profiling of different sized tuber revealed that palatable tubers are watery and very poor in puerarin (0.3 mg/g DW), diadzin (0.17 mg/g DW) and genistin (0.87 mg/g DW). The biggest tuber (~4.5 kg) contained highest amount of Puerarin (100.79 mg/g DW) obtained from one geographical region while the biggest tuber (~180 g) from another geographical region contained no detectable puerarin and high diadzin (10.4 mg/g DW). Further germination pattern of seeds revealed that a systematic management practice may increases the survival of seedlings up to 70%.

Keywords *Pueraria tuberosa* · Tuber · Isoflavonoids · Puerarin · Didzine · Geographical distribution

Introduction

Traditional, complementary and alternative medicine systems are being used and preserved in many countries including India due to dependencies of 80% population for healthcare remedies on the natural products obtained from medicinal plants (Bandyopadhyay 2018; Peltzer and Pengpid 2018). *Pueraria tuberosa* (Roxb.ex.Willd.) DC (family: Fabaceae), commonly known as "Vidarikand" is a perennial woody liana, with three leaflets, densely brown hairy 3–5 seeded pods and racemes of small purple- blue flowers. The pharmacologically important part of the plant is underground tuber, which grows in chain and varies in size and weight. The dried powder of tuber is used in various formulation of traditional medicine system as rejuvenating tonic, immune booster, antiaging, spermatogenic, aphrodisiac, diuretic, cardio tonic and galactogogue (Maji et al. 2014). A number of bioactive compounds have been identified in tubers, mainly isoflavones such as puerarin, diadzin, genistin, tuberosin and so on (Zhou et al. 2014). Dietary intakes of Isoflavonoids have a significant impact on human health and has the capability to decrease the risk of osteoporosis, attenuate the symptoms of menopause in women (Muangman and Cherdshewasart 2018; Yoshiara et al. 2018), possess anticancer (Zhao et al. 2018), anti-diabetic (Dong et al. 2018), anti-inflammatory, antioxidant (Liu et al. 2006), neuroprotective (Yang et al. 2012), wound healing (Kambhoja and Murthy 2007; Mukai et al. 2012), hypolipidemic (Takahashi et al. 2009), nootropic (Bagheri et al. 2011) activities. Recently daidzein has shown potential anti-inflammatory and cell inhibitory activity in endometriosis (Takaokaa et al. 2018; Yu et al. 2016). In a study on placebo controlled double blinds, Kudzu extract has been shown as a safe and potent adjunctive pharmacotherapy for reducing dependencies on alcohol (Penetar et al. 2015). Biotechnological efforts had been made to produce isoflavonoids from various species of *Pueraria.* This line of approach has progressed through optimization (manipulation of medium salts, precursors and plant growth regulators, designing of production medium) in

✉ Jaya Arora
jaya890@gmail.com

[1] Laboratory of Biomolecular Technology, Department of Botany, Mohan Lal Sukhadia University, Udaipur 313001, Rajasthan, India

cell cultures grown in shake flasks and bioreactor, selection of cell lines for high yields, cell mutations, biotransformations, manipulations of biosynthetic pathways, organized cultures, and lastly genetic engineering (Goyal et al. 2011; Sharma et al. 2011). Quantity of Isoflavonoids is significantly influenced by both the environmental as well as ecological factors. It has been observed that during the last few decades less attention had been paid for sampling and conserving the chemical diversity of medicinal plants and exploring the underlying genetic diversity found in plant population (Heywood 2002). Therefore, the objective of this study was to evaluate the Isoflavonoids content of various sized tubers of *P. tuberosa* distributed in various geographical regions of Southern Rajasthan. Study of seed germination pattern and survival rates of seedlings were also measured to establish a systematic conservation protocol.

Materials and methods

Collection of plant material

Regular field visits were made for collection of Pods containing seeds and tubers from various geographical area of Southern Rajasthan from the month of Feburary (Start of flowering) till the end of May. The plant identification was done by Prof. K.G. Ramawat, Department of Botany, MLSU, Rajasthan. The tubers of *P. tuberosa* were collected from Mount Abu, South Rajasthan, India (Fig. 1) and classified according to their diameter, namely PTRM1-4, corresponding to tubers with weight, 4654 mg, 759.54 mg, 243.88 mg and 118.68 mg respectively, fresh tuber of *P. tuberosa* collected from Panarwa, South Rajasthan were named PTRP1-2 corresponding to tubers with weight 120.60 mg and 180.80 mg; fresh stem of *P. tuberosa* collected from Panarwa was named PTPS1 with weight 17.50 mg.

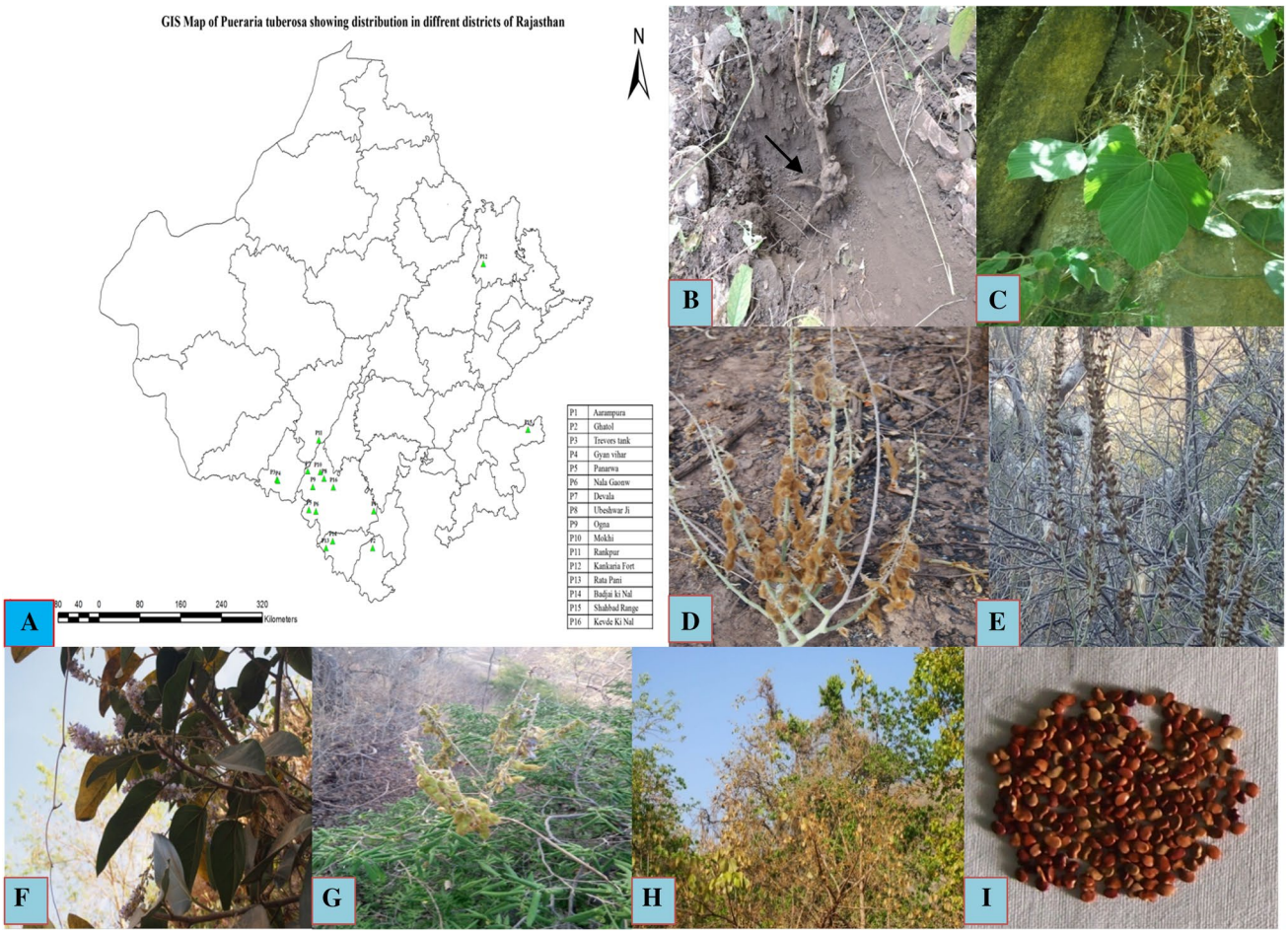

Fig. 1 GIS map and Different stages of *Pueraria tuberosa*: **a** GIS Map showing distribution in Southern Rajasthan, **b** plant with tuber, **c** shoot with leaves, **d** inflorescence with mature pods, **e** erect inflorescence with flower bud, **f** Inflorescence showing flowers, **g** unripe pods, **h** mature pods, **i** Seeds

Table 1 Evaluation of isoflavonoid contents from the tubers of *P. tuberosa* by HPLC method

S. No.	Tuber	Tuber Fresh weight (g)	Isoflavonoid content mg g^{-1} DW				Yield g kg^{-1}
			Puerarin	Genistin	Daidzin	Total isoflavonoids mg g^{-1}	
1.	PTRM1	4654.00	100.79 ± 3.25^{c}	–	16.00 ± 0.99^{c}	116.79^{d}	116.790
2.	PTRM2	759.54	5.582 ± 0.69^{b}	0.060 ± 0.02^{ab}	1.228 ± 0.23^{a}	6.959^{b}	6.959
3.	PTRM3	243.88	1.328 ± 0.74^{a}	0.0137 ± 0.07^{a}	0.123 ± 0.11^{a}	1.4757^{a}	1.476
4.	PTRM4	118.68	7.583 ± 0.43^{b}	–	1.670 ± 0.32^{a}	9.253^{b}	9.253
5.	PTRP1	120.60	0.361 ± 0.96^{a}	0.178 ± 0.06^{c}	0.874 ± 0.36^{a}	1.663^{a}	1.663
6.	PTRP2	180.80	–	1.595 ± 0.05^{d}	10.395 ± 1.20^{b}	10.434^{c}	10.434
7.	PTRS1	17.50	0.046 ± 0.16^{a}	0.117 ± 0.03^{bc}	1.713 ± 0.55^{a}	1.944^{a}	1.944
	F value		1.619	302.784	92.151	2.703	

Isoflavonoid content in tuber were analyzed for statistical significance and means in each column followed by same letters are not significantly different, while different letters in each column are statistically different according to Duncan's Multiple Range Test at $P < 0.05$

Chemicals and reagents

Puerarin, Diadzin and Genistin were purchased from Sigma-Aldrich, India with respective batch numbers of CASN 3681-99-0, CASN 552-66-9, CASN 529-59-9. The purity of each standard compound was above 98%. HPLC grade methanol and Acetonitrile were purchased from Himedia, India. For HPLC analysis Solvent A-0.0025% trifluoroacetic acid in water (E.Merck, India) and Solvent B- 80% acetonitrile (E.Merck, India) in solvent A were used. Water was prepared by a Milli-Q purification system. All other chemicals of analytical grade were used.

Sample preparation

The dry weight (DW) was determined by drying the tubers at 60 °C in an oven to a constant weight. For Isoflavonoids analysis of each sample, 0.2 g of the fine powder was suspended in 5 ml of 70% (v/v) methanol in mili-Q water for 12 h on room temperature (24–26 °C) with a speed 70 rpm on a test tube rotator (Abdos waves), centrifuged at 2000 rpm for 10 min and then the supernatant was collected and evaporated by Speed–vac sample concentrator (model SPD 111 V,Thermo Savant, USA). For HPLC analysis all the extracts were redissolved in HPLC grade methanol, sonicated for 10 min by sonicator (Sonar), filtered through nylon syrings filter (0.2 µm, 4 mm, National, USA) and transferred in 300 µl sample vials.

High performance liquid chromatography analysis of tubers

The HPLC system used for the separation of compounds was equipped with a PerkinElmer™ LC pump Series 200, a UV detector (N2910383, Perkin Elmer) controlled with "PerkinElmer®"s TotalChrom® Chromatography Data System (CDS) software". Separation was accomplished on a Perkin Elmer Series 200 HPLC Peltier Column (5 µm) RP-18 column protected by a gurad column of the same material. The HPLC analysis was performed using solvent system as: Solvent A—0.0025% trifluoroacetic acid in water; Solvent B—80% acetonitrile in solvent A. The mobile phase consisted of solvent (A) and (B). The step gradient solvent programme used was as follows: 0–2 min: 85% A and 15% B; 2–5 min: 85% A and 15% B: 5–15 min: 80% A and 20% B; 15–20 min: 50% A and 50% B; 20–30 min: 40% A and 60% B; 30–35 min: 30% A and 70% B; 35–45 min: 20% A and 80% B; 45–48 min: 0% A and 100% B: 48–50 min: 0% A and 100% B: 50–55 min: 85% A and 15% B. Separation was performed at a flow rate of 1.0 ml/min and chromatographic Peaks wear monitored at 254 nm (Goyal et al. 2011; Krisa et al. 1999). The analyses of the samples were run in triplicate, identification being made by comparing the retention times and quantified by using standard curves for the peak area of the isoflavonoid standards.

Study of seed germination pattern and conservation techniques

Germination of seeds was studied by a complete randomized block design with six replicates. Seeds were sown at a depth of 0.5 cm in DCS Reusable Seedling (Germination) Tray with round 98 cells plant container, filled with soil (loamy sand; 60% sand, 25% silt, and 15% clay). The complete experiment was carried out from the month of August–October in in vivo conditions with moisture ~ 62–67% and temperature ~ 27–32 °C. Seedling emergence was recorded weekly up to 60 days. A seed was considered germinated till the appearance of two cotyledonary leaves. Total healthy seedlings, in germinated seed was considered for survival rate after 60 days. Results were pooled to determine percent

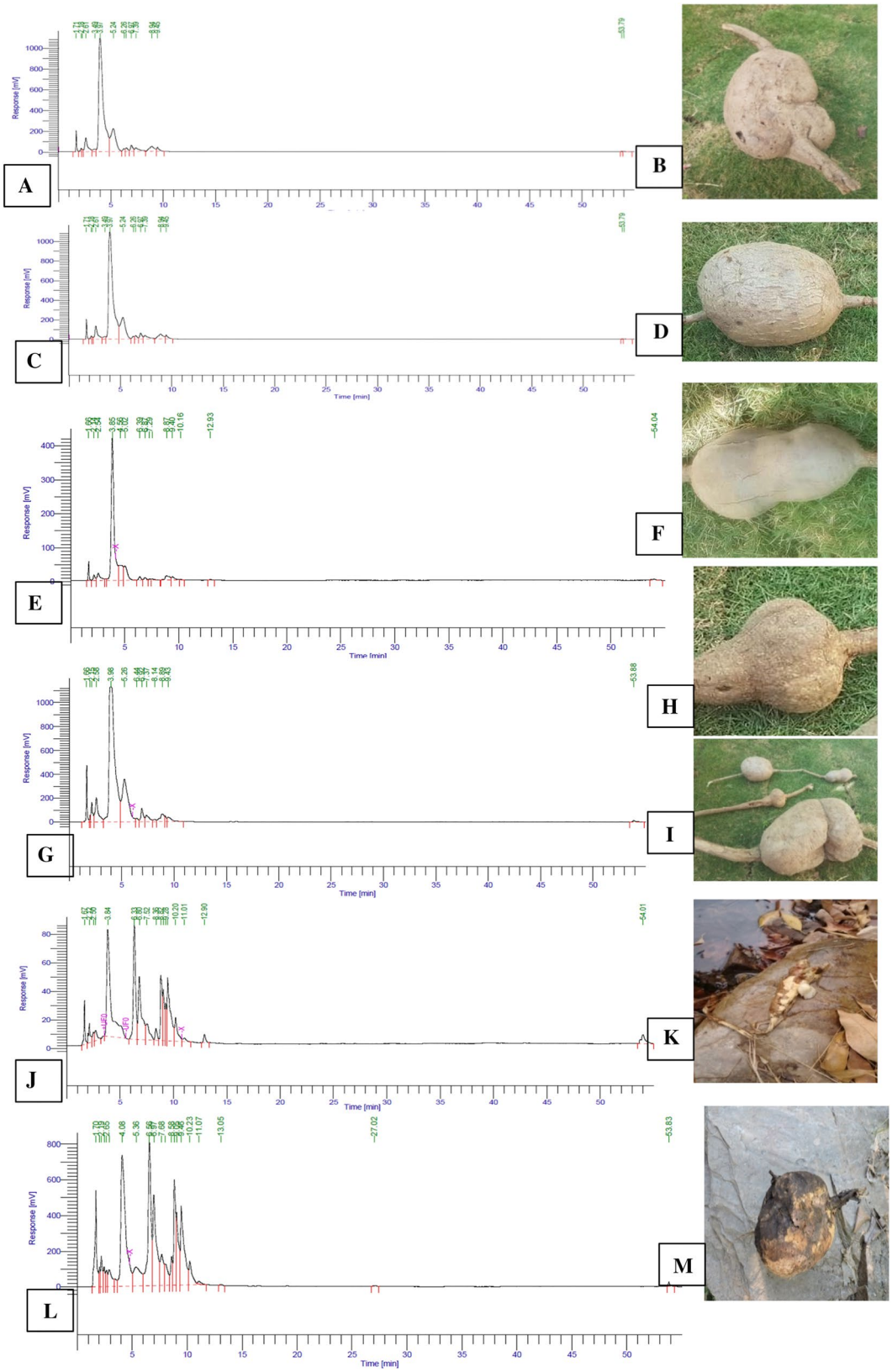

Fig. 2 Fractionation of the isoflavone compounds of 4654 g tuber (**a**, **b**), 759.54 g tuber (**c**, **d**), 243.88 g tuber (**e**, **f**), 118.68 g tuber (**g**, **h**), difference in tuber size (**i**); collected from Mount Abu hills, Sirohi, Rajasthan, India by HPLC at 254 nm. Fractionation of the isoflavone compounds of 120.60 g tuber (**j**, **k**) and 180.80 g tuber (**l**, **m**) collected from Panarwa region of Jhadol, Udaipur, Rajasthan, India by HPLC at 254 nm

germination and percent survival rate. To increase the germination percent various techniques were used.

Mechanical scarification

Ten seeds were 20 min rubbed in groups in between the sand papers to break the hard testa and seeds were kept for germination in DCS Reusable Seedling (Germination) Tray Round 98 Cells Plant Container.

Chemical scarification

The seeds were treated with conc. H_2SO_4 separately for 05 min, 10 min, 15 min, 20 min and 25 min respectively and kept for germination in DCS Reusable Seedling (Germination) Tray Round 98 Cells Plant Container.

Statistical analysis

The isoflavonoid contents from the tubers of *P. tuberosa* were analyzed for statistical significance by Duncan analysis of variance at the significance level of $P < 0.05$ using SPSS 16.0 software.

Results and discussion

Geographical distribution and GIS map of plant

Pueraria tuberosa was observed that woody liana is distributed in the form of dispersed tubers and the shoot was with green leaves was found only at shady moist places while open sunny areas have only dried stem with no leaves. In the Mount Abu a cut open rock in front of Gyan Vihar revealed how a single woody liana is spread over a wide area. This kind of distribution also revealed that in nature harvesting of same sized tubers is not possible due to uneven distribution. Anthropogenic activities are also disturbing *P. tuberosa* distribution in nature. A GIS map with the software (ArcGIS) was created showing its geographical distribution in Sothern Rajasthan (Fig. 1).

High performance liquid chromatography analysis of tubers

HPLC profiling of different sized tuber revealed that palatable tubers are watery in nature and very poor in three important secondary metabolites viz Puerarin (0.3 mg/gDW), Diadzin (0.17 mg/gDW) and Genistin (0.87 mg/gDW). The biggest tuber (~4.5 kg) contained highest amount of Puerarin (100.79 mg/g DW) obtained from one geographical region while the biggest tuber (~180 g) from another geographical region contained no detectable puerarin and high diadzin (10.4 mg/gDW), (Table 1, Fig. 2). So according to environmental conditions available the quality and quantity of isoflavones both are variable. A difference in anthocyanin content of *Lycium ruthenicum* Murray from different geographical regions of China was obtained (Wang et al. 2018). *Urera aurantiaca* has shown difference in polyphenol contents from two different provenance of Argentina (Marrassini et al. 2018). The *Artemisia annua* L. distributed in Northern China shown high arteannuin B and artemisinic acid content (Zhang et al. 2017). The herb also showed strong adaptability to different environmental conditions (Zhang et al. 2008). The phenolics, saponins and flavonoids contents in the flower buds of *Aralia elata* were affected by different geographical origins of China (Qi et al. 2018). The size of tuber is also important for accumulation of secondary metabolites. As the dry weight of tuber increases aconitine content decreased in *Aconitum japonicum* tuber. There is also a spatial distribution of aconitine in apical and basal parts of tuber (Ishisaki et al. 2018). In the metabolic profiling analysis done by Jiang et al. (2006), the *Zingiber officinale* samples derived from different origins showed no qualitative differences in major volatile compounds, although they did show some significant quantitative differences in non-volatile composition, particularly regarding the content of [6]-, [8]-, and [10]-gingerols, the most active anti-inflammatory components in this species.

Study of seed germination pattern and conservation techniques

Effect of environmental conditions on tuber size and its content is so prominent that a systematic study of seed germination pattern and conservation techniques is essential for this woody liana. This type of systematic study for *P. tuberosa* has not done yet. Seeds of *Pueraria* take time in germination after sowing ranging from 4 to 28 days. Seeds of *P. lobata* have been shown great dormancy with only 6–17% seed germination. Due to hard seed coat, water absorption is low and various methods have been used to break dormancy (Susko et al. 2011). In control conditions with no treatments 28% seed germination was observed in *P. tuberosa* which increased up to 40% on treatment with conc. H_2SO_4 for 25 min, however 15 min treatment of conc. H_2SO_4 also shown 38% germination. Among various treatments highest germination percentage and seedling survival (80%) was observed in Conc. H_2SO_4 treatment for 15 min (Figure 3). Though the seed germination pattern was increased in different treatments but survival rate of seedlings was not uniform for all treatments. *Leucaena leucocephala* showed best efficiency in overcoming seed dormancy by immersion of seeds in concentrated H_2SO_4 for 20 min, with 93.0% of viable seed germination (Bichoff et al. 2018). In *Verbascum calycosum* increased germination after GA_3 application and mechanical

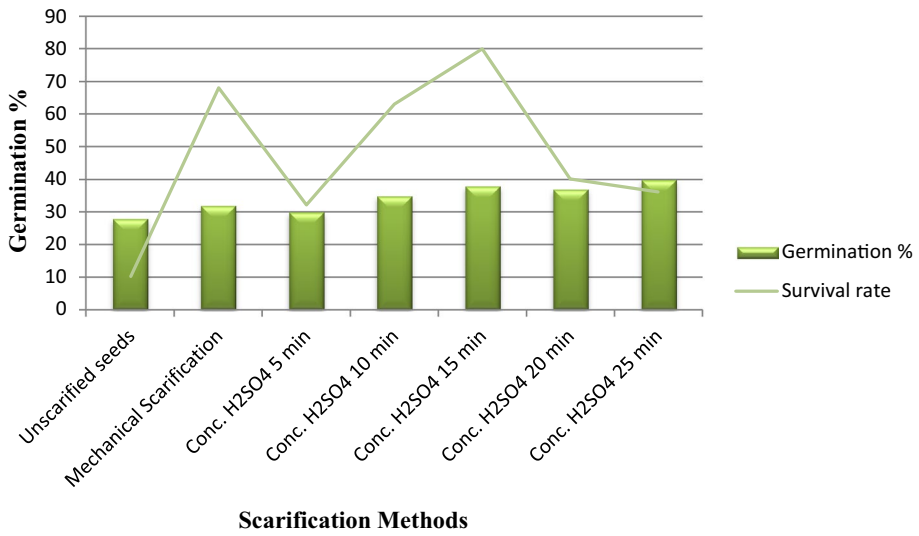

Fig. 3 Study of seed germination pattern and survival rate of *P. tuberosa* seeds under different treatments (Control, Mechanical and Chemical Scarification)

scarification but the highest germination index was obtained at cold stratification of + 4 °C and − 20 °C (Hilooglu et al. 2018). There is a lot of need of more trials and combinations for understanding dormancy breaking method of germination and increase in survival rates of seedlings.

Conclusions

In the present study correlation of tuber size, its geographical distribution with isoflavonoids synthesis has been established very well. An efficient modified protocol for HPLC–UV detection has been established. All three reference compounds viz puerarin, diadzin and genestin have been found in all samples. It has been concluded that increase in tuber size is directly correlated with increased isoflavonoids content. A ~ 4.5 kg tuber can yield 116.79 mg/g DW total isoflavonoids in which the main marker compound Puerarin is present at the concentration of 100.79 mg/g DW, which has not been reported in any study. From commercial aspects it is a quite high content. It was also observed that at tender stage when tubers are eatable the total content of all three metabolites was very low still with increase in weight there was accumulation of diadzin but no puerarin content was obtained. So it is concluded that when tuber becomes dried and there is increase in weight there is increase in puerarin content while fresh and watery tuber are very low in puerarin content and are of no commercial use. As the tuber exploitation is directly correlated with plant death seed germination pattern was also studied in nature. Systematic and proper environmental conditions give 40% germination and 32% survival of seedlings and tuber formation was observed in 60 days old seedlings, which is not possible to monitor in natural conditions due to various limiting factor. A systematic conservation practice and tuber harvesting can be done form industrial point of view.

Limitations of the study

In the current study naturally plant tubers were found many feet under the soil and the plant is on the verge of extinct in Southern Rajasthan, thus, we cannot collect same size tuber. Furthermore, stage of tuber we did explain, which could have confirmed our findings.

Acknowledgements The authors acknowledge the support of the Department of Science & Technology (DST), New Delhi, India for the Grant of Project (No.SR/WOS-A/LS-231/2016 Dated 18-07-2016) to Ms Bhanupriya Kanthaliya under Wos A scheme (Woman Scientist) for conducting this work. The authors also acknowledge support of Prof. Seema Jalan, Head, Department of Geography, MLSU, Udaipur, India in preparing GIS map.

Compliance with ethical standards

Conflict of interest There are no conflicts of interest.

References

Bagheri M, Joghataei MT, Mohseni S, Roghani M (2011) Genistein ameliorates learning and memory deficits in amyloid b (1–40) rat model of Alzheimer's disease. Neurobiol Learn Mem 95:270–276

Bandyopadhyay D (2018) Protection of Traditional Knowledge and Indigenous Knowledge. In: Bandyopadhyay D (ed) Securing our natural wealth: a policy agenda for sustainable development india and its neighbouring countries. Springer, Singapore, pp 59–70

Bichoff RS, Okumura RS, Oliveira RS et al (2018) Overcoming seed dormancy and evaluation of viability in leucaena leucocephala [online]. Aust J Crop Sci 12:168–172

Dong S, Zhang M, Niu H, Jiang K, Jiang J, Ma Y et al (2018) Upregulation of UDP-glucuronosyltransferases 1a1 and 1a7 are involved in altered puerarin pharmacokinetics in type II diabetic rats. Mol 23:E1487

Goyal S, Sharma V, Ramawat KG (2011) Marked effect of *Cuscuta* on puerarin accumulation in cell culture of *Pueraria tuberosa* grown in shake flasks and a bioreactor. Plant Biotechnol Rep 5:121–126

Heywood VH (2002) The conservation of genetic and chemical diversity in medicinal and aromatic plants. In: Şener B (ed) Biodiversity. Springer, Boston, pp 13–22

Hĭlooğlu M, Sözen E, Yücel E, Kandemir A (2018) Chemical applications, scarification and stratification effects on seed germination of rare endemic Verbascum calycosum Hausskn. ex Murb. (Scrophulariaceae). Not Bot Horti Agrobot Cluj Napoca 46:376–380

Ishisaki M, Kawaguchi K, Takahashi S, Wada Y (2018) Relationship between tuberous root size and aconitine alkaloids content and the distribution of aconitine alkaloids in tuberous roots of aconite plants (*Aconitum japonicum* Thunberg). Jpn J Crop Sci 87:76–82

Jiang H, Xie Z, Koo HJ, McLaughlin SP, Timmermann BN, Gang DR (2006) Metabolic profiling and phylogenetic analysis of medicinal *Zingiber* species: tools for authentication of ginger (*Zingiber officinale* Rosc.). Phytochemistry 67:1673–1685

Kambhoja S, Murthy KRK (2007) Wound healing and anti-inflammatory activity of *Pueraria tuberosa* (Roxb Ex wild) DC. Biomed 2:229–232

Krisa S, Larronde F, Budzinski H, Decendit A, Deffieux G, Merillon JM (1999) Stilbenes production by Vitis vinifera cell suspension cultures: methyl jasmonate induction and 13C biolabeling. J Nat Prod 62:1688–1690

Liu P, Zhao YX, Zhang Y (2006) Clinical observation of daidzein intervention on serum inflammatory factors in senile patients with coronary heart disease. Chin J Integr Med 26:42–45

Maji AK, Pandit S, Banerji P, Banerjee D (2014) *Pueraria tuberosa*: a review on its phytochemical and therapeutic potential. Nat Prod Res 28:2111–2127

Marrassini C, Peralta I, Anesini C (2018) Comparative study of the polyphenol content-related anti-inflammatory and antioxidant activities of two Urera aurantiaca specimens from different geographical areas. Chin Med 13:22

Muangman V, Cherdshewasart W (2018) Clinical trial of the phytoestrogen-rich herb, *Pueraria mirifica* as a crude drug in the treatment of symptoms in menopausal women. Siriraj Med J 53:300–309

Mukai K, Nagasawa M, Nakamura E et al (2012) The effect of isoflavone-daidzein oral medication on cutaneous wound healing in female ovariectomized mice. Struct Func 10:94–100

Peltzer K, Pengpid S (2018) Prevalence and determinants of traditional, complementary and alternative medicine provider use among adults from 32 countries. Chin J Integr Med 24:584–590

Penetar DM, Toto LH, Lee DYW, Lukas SE (2015) A single dose of kudzu extract reduces alcohol consumption in a binge drinking paradigm. Drug Alcohol Depen 153:194–200

Qi M, Hua X, Peng X, Yan X, Lin J (2018) Comparison of chemical composition in the buds of *Aralia elata* from different geographical origins of China. R Soc Open Sci 5:180676

Sharma V, Goyal S, Ramawat KG (2011) Increased puerarin biosynthesis during in vitro shoot formation in *Pueraria tuberosa* grown in growtek bioreactor with aeration. Physiol Mol Biol Plants 179:87–92

Susko DJ, Mueller JP, Spears JF (2011) An evaluation of methods for breaking seed dormancy in kudzu (*Pueraria lobata*). Can J Bot 79:197–203

Takahashi Y, Odbayar TO, Ide T (2009) A comparative analysis of genistein and daidzein in affecting lipid metabolism in rat liver. J Clin Biochem Nutr 44:223–230

Takaokaa O, Moria T, Ito F, Okimura H, Kataoka H, Tanaka Y et al (2018) Daidzein-rich isoflavone aglycones inhibit cell growth and inflammation in endometriosis. J Steroid Biochem Mol Biol 181:125–132

Wang Z, Yan Y, Nisar T, Zou L, Yang X, Niu P, Guo Y et al (2018) Comparison and multivariate statistical analysis of anthocyanin composition in *Lycium ruthenicum* Murray from different regions to trace geographical origins: the case of China. Food Chem 246:233–241

Yang SH, Liao CC, Chen Y, Syu JP, Jeng CJ, Wang SM (2012) Daidzein induces neuritogenesis in DRG neuronal cultures. J Biomed Sci 19:1–13

Yoshiara L, Madeira T, de Camargo A, Shahidi F, Ida E (2018) Multistep optimization of β-glucosidase extraction from germinated soybeans (*Glycine max* L. Merril) and recovery of isoflavone aglycones. Foods 7:110

Yu J, Bi X, Yu B, Chen D (2016) Isoflavones: anti-inflammatory benefit and possible caveats. Nutrients 8:361

Zhang R, Zhao M, Han S (2008) Study on contents of artemisinin in *Artemisia annua* L. introduced from different provenances. Chem Industry Forest Prod 28:83–87

Zhang X, Zhao Y, Guo L, Qiu Z, Huang L, Qu X (2017) Differences in chemical constituents of *Artemisia annua* L. from different geographical regions in China. PLoS One 12:e0183047

Zhao YR, Du LD, Zhang L, Du GH (2018) Puerarin. In: Du GH (ed) Natural small molecule drugs from plants. Springer Nature, Singapore Pte. Ltd. People's Medical Publishing House, Singapore

Zhou YX, Zhang H, Peng C (2014) Puerarin: a review of pharmacological effects. Phytother Res 28:961–975

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.