J. Hortic. Sci.
Vol. 19(2), 2024

https://doi.org/10.24154/jhs.v19i2.2206

Original Research Paper

Prefatory studies on identification of anti-diabetic factor in callus cultures of *Momordica cymbalaria*

Raghavendra S.[1*], Raghavendra G.[2], Jagdeesha R.C.[2], Kiran Kumar K.C.[2] and Anupama G.[2]

[1]Department of Biochemistry, Division of Plant Science,
College of Agriculture, Navulie, Shivamogga - 577 204, Karnataka, India
[2]College of Horticulture, Udayanagiri Campus, University of Horticultural Sciences,
Bagalkot 587 104, Karnataka, India
[*]Corresponding author Email: raghavendras@uahs.edu.in

ABSTRACT

Momordica cymbalaria is an underutilized folk vegetable crop in the northern region of Karnataka, India. Its fruit is consumed as diet, which has antidiabetic and hypolipidemic effect. A 17 kDa protein known as M.cy protein has been identified as anti-hyperglycaemic peptide present in fruit. The crop duration is from June to September and propagated through tuber only, while, seeds are dormant. The callus induction was achieved from explants enriched with 2, 4 D (3 mg L^{-1}) and BAP (0.2 mg L^{-1}). Crude protein was extracted from the callus and the seed using extraction buffer (50 mM Sodium acetate buffer, pH 4.8). The extracted protein was subjected to SDS-PAGE. The protein ladder used in the present study was pre-stained with three color, covering wide range molecular weights from 10 to 245kDa. The three bands roughly identified around 10 kDa to 25 kDa named as PF1 (Protein fraction), PF2 and PF3 were isolated from the gel and were subjected to *in vitro* anti-diabetic assay by á-amylase enzyme assay kit and the inhibitory activity was expressed as percentage of inhibition. All the protein fractions PF1, PF2 and PF3 showed positive for the á-Amylase enzyme assay indicating that there are other peptides present in the callus apart from the M.cy protein present in the seeds that have anti hyperglycaemic properties.

Keywords: Anti-hyperglycemic properties, *Momordica cymbalaria*, protein fraction, á-Amylase enzyme

INTRODUCTION

Plant foods play a vital role in cultural system of tribal people for reducing various disorders and found to be a good source of secondary metabolites (Raghavendra et al., 2021). As research interest is now shifted from secondary metabolites to bio-molecules, plants are now under exploration for the presence of proteins and bioactive peptides for the disease management (Shital *et al*., 2020). The use of medicinal plants for the treatment of diabetes dates back from the Ebers papyrus of about 1550 B.C. It antiquated used in the distinctive medicinal systems for a long time for the treatment of diabetes mellitus (Jeyadevi et al., 2012). The genus *Momordica* native to the tropic (Robinson & Decker-Walters, 1997) comprises about 59 species (Schaefer & Renner, 2010) distributed chiefly in Africa and Southeast Asia (de-Wilde & Duyfjies, 2002). Karnataka, Madhya Pradesh, Maharashtra, Andhra Pradesh and Tamil Nadu are the regions wherein these genera are found in plentiful. *M. cymbalaria* is a climbing annual or a perennial herb with slender, scandent, branched, striate stem. The leaves are orbicular-reniform, fruits are 20-25 mm long, pyriform and the seeds are 4.6 long, ovoid shaped, smooth and shiny. Flowers are unisexual. The roots are woody, tuberous and perennial. Numerous pharmacological actions of *M. cymbalaria* have been studied in animal models for anti-diabetic activity (Mahesh et al., 2018), cardioprotectivity (Koneri et al., 2007), hepatoprotective activity (Kumar et al., 2008), nephroprotectivity (Kumar et al., 2011), and reproductive health (Abbirami et al., 2021). The available literature shows that there are more than 400 plant species showing hypoglycemic activity and though some of the plants are believed in the indigenous systems of medicine for their activities, it remains to be scientifically well-known. *M. cymbalaria* not only exhibits anti-hyperglycaemic activity but also anti-diabetic activity with the possible involvement in regeneration of β cells and by increasing insulin levels. (Rao et al., 2003). The present study is a preliminary attempt to isolate a

bioactive compound, a possible peptide responsible for antidiabetic activity in callus cultures of *Momordica cymbalaria*.

MATERIALS AND METHODS

Callus induction

The planting material were collected from the agriculture field of Kaladagi village, Bagalkot district, Karnataka and sown in College of Horticulture Bagalkot. Leaves and stem were used for callus induction. Explants were surface sterilized with 0.1% mercuric chloride and washed with water for four times to remove the traces of mercuric chloride and aseptically trimmed (1.5 to 2.0 cm) and inoculated onto MS medium supplemented with sucrose (30 g L^{-1}), 0.2 mg L^{-1} BAP and varying concentrations from 0.5 mg L^{-1} to 4 mg L^{-1} each of 2, 4-D, NAA and Kinetin for callus formation. The media were solidified with 0.8% (w/v) agar and adjusted to pH 5.8 prior to autoclaving at 121°C for 20 min. Cultures were maintained at 26±2°C under a 16-h light/8-h dark photoperiod.

Isolation and identification of anti-diabetic factor

Extraction of protein

The extraction of protein was carried out as described by Maddirela et al. (2010) with appropriate modifications. All the steps were carried out at 4°C. Crude protein was extracted using extraction buffer (50 mM Sodium acetate buffer, pH 4.8). 50 mg callus were homogenized in 5 mL of 50 mM Sodium acetate buffer, pH 4.8 and the homogenate was filtered and centrifuged at 12,500 × g (REMI, India) for 20 min at 4°C, the supernatant was collected and the total protein was estimated by Bradford method (1976). The crude protein sample so extracted was used as protein source. In parallel the protein was also extracted from the seeds of *Momordica cymbalaria* for the confirmation of the presence of anti-diabetic peptide (Maddirela et al., 2010).

SDS PAGE analysis

The standard protocol followed for SDS-PAGE was as described by Laemmli et al. (1970) using 5% stacking gel and 10% separating gel with slight modification. The separation buffer contained: 0.5 M Tris-HCl (pH 6.8), 2.5% SDS, 10% glycerol and 5% 2-mercaptoethanol. Bromophenol Blue was added to the sample buffer as tracking dye. Approximately 8 µg of protein was loaded into each well. In order to check the reproducibility of the method two separate gels were run under similar electrophoretic conditions. The molecular weights of the seperated polypeptides were determined by using molecular weight protein standards "MBT 092" from Himedia, pre-stained with three-color awning wide range molecular weights from 10 to 245 kDa.

Protein isolation from polyacrylamide gels

The lower 3 peptide bands (Fig. 2) roughly identified around 10 kDa to 25 kDa named as PF1 (Protein fraction), PF2 and PF3 were localized, cut with a razor blade and washed (three times for 5 mins) with 2 mL of 250 mM Tris buffer/250 mM EDTA, pH 7.4, followed by three rinses of 5 min with distilled water (Retamal et al., 1999). The water was removed and the gel slices were chopped finely (in pieces of 2-5 mm) then 1 mL of buffer containing 50 mM Tris HCl, 0.1 mM EDTA and 150 mM NaCl was added (the ratio of buffer volume to gel piece volume was approximately 2:1). The chopped gel along with buffer was incubated in a rotary shaker overnight at 30°C. Later, centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was carefully then pipette out into an eppendorf tube.

Anti-diabetic assay by α-amylase enzyme assay

In vitro amylase inhibition was studied by the method of Bernfeld (1955) with appropriate modifications. A total of 500 µl of sample (seed, crude callus and eluted protein bands) and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-Amylase solution (0.5 mg/mL) were incubated for 10 minutes at 25°C. After pre-incubation, 500 µl of 1% starch solution in 0.02 M sodium phosphate buffer was added to each sample at 5s interval. This reaction mixture was then incubated for 10 minutes at 25 p C. 1.0 mL of DNS was added to stop the reaction. These tubes were then incubated in boiling water bath for 5 minutes and cooled to room temperature. Finally, this reaction mixture was again diluted by adding 10 mL distilled water and the absorbance was measured at 540 nm.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \text{ X } 100$$

LC-MS analysis

The supernatant obtained from the SDS PAGE were subjected to LC (Waters Acquity UPLC) with a quaternary pump and a diode array detector (DAD). The column is coupled with an MSD Ion Trap XCT mass spectrometer (Waters Xevo G2-XS QTof) equipped with an electrospray ionization interface. Samples (200 µL) were injected onto a BEH C-18 column (50 X 2.1, 1.7 µ). The mobile phase composed of A- 0.1% formic acid in water and B – Methanol with a flow rate of 0.4 mL^{-min} following the gradient: 98–2% A linear (0–8 min), 2–98% B linear (0–8 min). This was followed by a 15 min equilibrium period with initial conditions. Spectra were recorded in negative and positive ionization mode between m/z 50 and 1200.

RESULTS AND DISCUSSION

Collection of plant materials and callus induction

The efficacy of callus initiation be contingent on many factors (Magdalena et al., 2020). In the present study, callus induction was observed in both stem and leaf explants on the medium supplemented with either auxins alone (2, 4-D/NAA/Kinitine) or low cytokinin to high auxin concentrations (data not shown) as mentioned in the materials and methods. The induced callus proliferated and covered the entire surface of the explants after 2-3 weeks of culture period. The maximum per cent of callus induction was noticed in leaf segment followed by stem segment. Medium supplemented with 2,4-D (3 mg L^{-1}) and 6-BAP (0.2 mg L^{-1}) combination found to be optimal for callus induction and proliferation when compared to NAA and Kinetin for both the explants. Callus induced with 2,4-D and NAA was white-friable and fast growing (Fig. 1), whereas, the callus induced with Kinetin combination was green-compact (Fig. 1). Callus induction through an auxin–cytokinin combination has been reported for several plants including *Pedalium murex* (Saravanan et al., 2007), *Brassica oleraceae* (Munshi et al., 2007), *Solanum tuberosum* L (Ahmet Metin & Sezai, 2015), *Nelumbo nucifera* (Xianbao et al., 2020). Nikam et al. (2008) has reported the successful influence of aforementioned combination on induction of callus in *Momordica cymbilaria*.

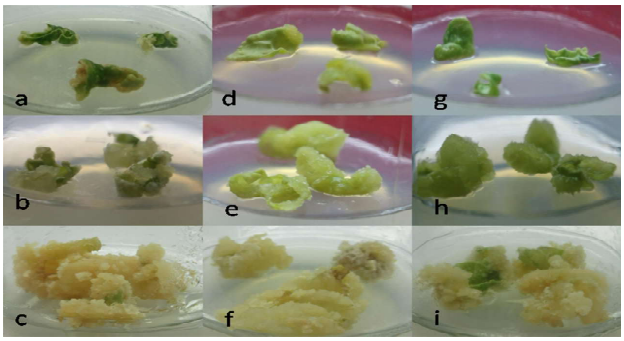

Fig. 1 : Different stages of callus initiation

a, b and c: explants inoculated on media containing 2,4D/ BAP (3/0.2 mgL^{-1}) after 8 days of initiation, 15 days after initiation and 25 days after initiation respectively; d, e and f: explants inoculated on media containing NAA/BAP (2.5/0.2 mgL^{-1}) after 10 days of initiation, 20 days after initiation and 30 days after initation respectively; g, h and i: explants inoculated on media containing Kinetin/BAP (2.5/0.2 mgL^{-1}) after 10 days of initiation, 20 days after initiation and 30 days after initiation

SDS PAGE analysis

Electrophoretic pattern of callus proteins in the present study are presented in Fig. 2. Some bands obtained on the gel were too faint and were not visible in the photograph. The protein bands were separated according to their molecular weight *i.e.* higher molecular weight proteins were in the upper region and the lower molecular weight proteins were in the lower region of the gel. The three bands roughly identified around 10 kDa to 25 kDa named as PF1, PF2 and PF3 were isolated from the gel and were subjected to *in vitro* anti-diabetic assay by α-amylase enzyme assay.

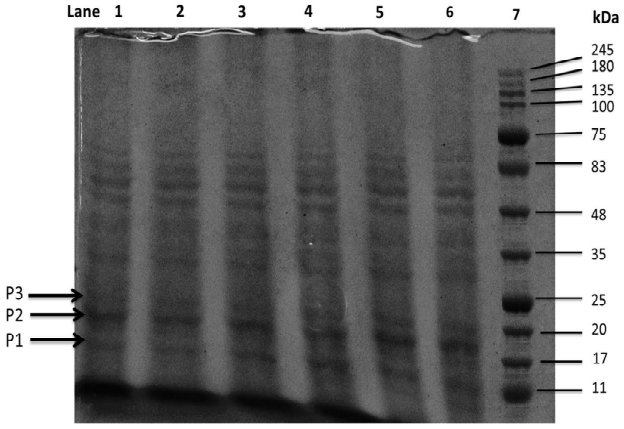

Fig. 2 : Protein banding pattern in SDS-PAGE

Lane 1-6 shows coomassie brilliant blue stained electrophoretogram (on 12% SDS-PAGE) of sodium acetate extract of callus culture showing several different protein bands; Lane 7 shows banding of standard protein markers

α-amylase enzyme inhibitory assay

The inhibitory activity was expressed as percentage of inhibition. Seed crude protein, crude callus protein and the three protein fractions PF1, PF2 and PF3 showed positive for the α-amylase enzyme inhibitory assay. Per cent inhibition was higher in the peptide proteins fractions followed by seed protein and callus protein. The protein fraction PF1, PF2 and PF3 showed 40.8, 40.2 and 40.6% inhibition respectively whereas seed protein showed 36.9% and crude callus protein showed 17.2%. Many peptides are proclaimed from different plants for their anti-diabetic activity (Sarmadi et al., 2012; Vaštag et al., 2014; Arise, 2016; Gonzalez Garza et al., 2017; Wang et al., 2018).

LC-MS analysis

LC-MS chromatogram of the separated bands from SDS PAGE and seed crude protein extract is shown in Fig. 3. The result showed the presence of molecular mass of 17 kDa peptide in the seed protein extract (Fig. 3a) which was earlier reported by Maddirela et al. (2010). The callus peptides isolated when subjected to mass spectrometry showed the absence of 17 kDa peptide. (Fig. 3b,c,d). The ionization of the PF1 didn't show the presence of 17 kDa protein. The PF2 samples ionized negatively and positively. Neither of the charges show the ionization at 17 kDa proving the absence of the peptide that is present in the seed. Fig. 3d showed the ionization of sample PF3 both negatively and positively. The mass of the peptide didn't ionize at 17 kDa indicating the absence of 17 kDa peptide.

Various peptides have been reported from diverse plants that have anti-diabetic activity (Shital et al., 2020). Exultance of hormone-like peptides along with insulin-like peptide in several plants' species support

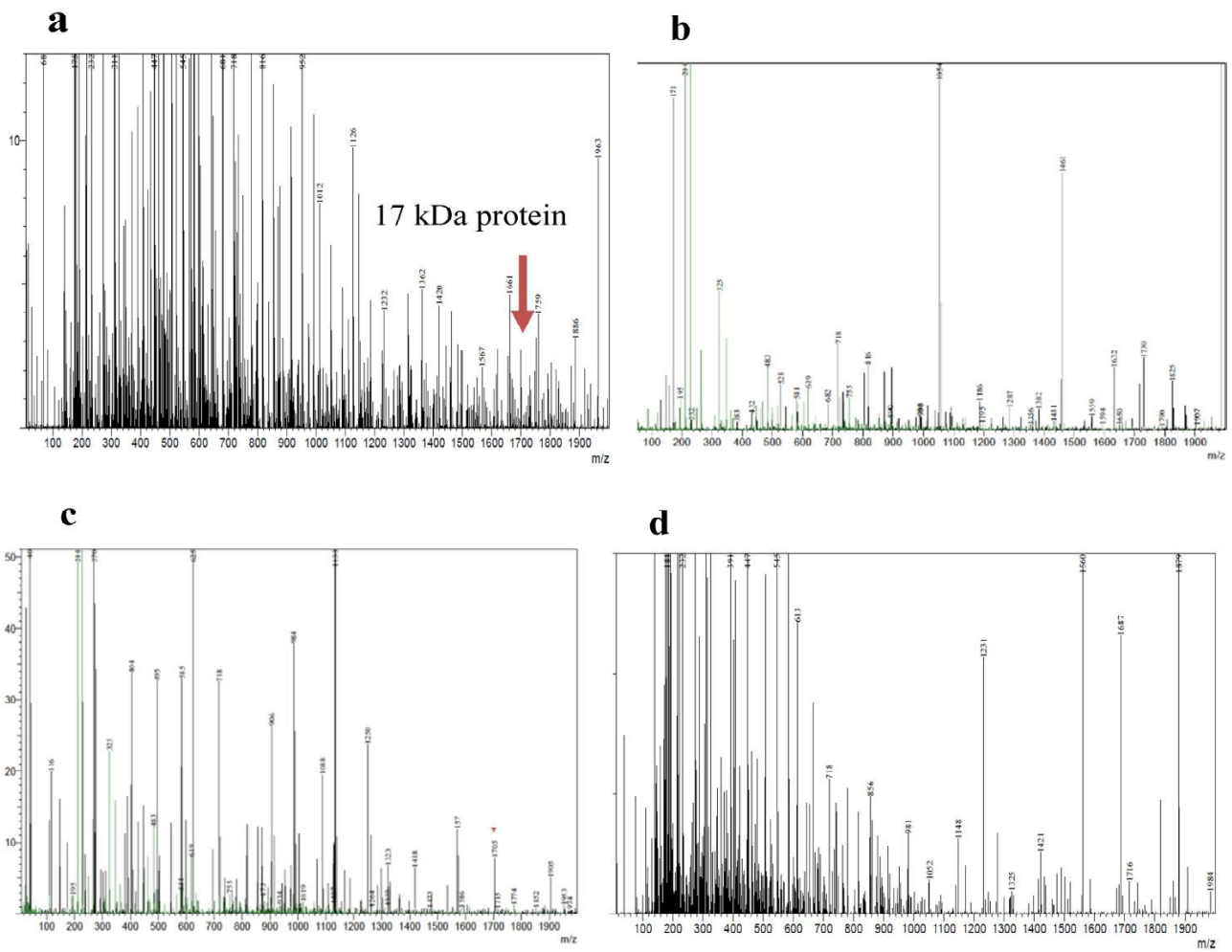

Fig. 3 : Mass spectrometry study of the separated bands from SDS PAGE, and seed crude protein extract
a. Seed crude protein b., PF1, c. PF2, d. PF3

this supposition, it is contemplated that the signaling peptides in plants existed from about 1 billion years ago (Collier et al., 1987; Fukushima et al., 1976; Leroith et al., 1985; Morley et al., 1980). In the present study, the MS data depicted the result as follows, though the 17 kDa was absent in all the three peptide fractions of the callus, there was inhibition of the α-amylase enzyme proving the presence of anti-diabetic factor. The peptides were more efficient in inhibiting the α-amylase activity when compared with the seed or crude callus extract. This indicates that that there are other peptides present in the callus cultures of *Momordica cymbalaria* apart from the M.cy protein present in the seeds that have anti-hyperglycemic properties.

CONCLUSION

Apparently, most of the bioactive peptides accessible for the treatment of diabetes have arose from the synthetic route or derivative from recombinant technology, which further adds in the inclusive cost of the treatment. To make peptide therapy fiscal, there is a demand to explore plant-based biomolecules. In the present study, we have succeeded in a preliminary attempt to prove that callus of *Momordica cymbalaria* have anti-diabetic peptide. However, the specific bioactive peptide responsible for anti-diabetic activity was not identified. Seeds of the plant has the well-studied information of the presence of M.cy protein that has anti diabetic activity. We tried for the same in the callus, but we originate unlike peptides apart from M.cy protein studied earlier. And also, we have attempted only fundamental *in vitro* studies leaving substantial opportunity for further studies in *in silico* and characterization of these peptides etc. In conclusion, this is the first report showing presence of antidiabetic peptides in the callus cultures of *Momordica cymbalaria*.

ACKNOWLEDGMENT

The authors are thankful to University of Horticultural Sciences, Bagalkot, and College of Horticulture, Bagalkot for their support during the research.

REFERENCES

Abbirami, E., Siva, D., Abinaya, S., Sooraj, R., Dinesh, K. L., Guna R., & Sivasudha, T. (2021). *Momordica cymbalaria* improves reproductive parameters in alloxan induced male diabetic rats. *3 Biotech*, *11*, 76. https://doi.org/10.1007/s13205-020-02612-8

Ahmet, M. K., & Sezai, E. (2015). Callus induction, shoot proliferation and root regeneration of potato (*Solanum tuberosum* L.) stem node and leaf explants under long-day conditions. *Biotechnology & Biotechnological Equipment*, *29*(6), 1075-1084. http://dx.doi.org/10.1080/13102818.2015.1077685

Arise, R. O. (2016). *In vitro* antioxidant and α-amylase inhibitory properties of watermelon seed protein hydrolysates. *Environmental and Experimental Biology*, *14*, 163–172. http://doi.org/10.22364/eeb.14.23

Bernfeld, P. (1955). "Amylases α and β" In: SP Colowick and NO Kaplan Eds., Methods in Enzymology. Academic Press, New York.149-158. https://doi.org/10.1016/0076-6879(55)01021-5

Collier, E., Watkinson, A., Cleland, C. F., & Roth, J. (1987). Partial purification and characterization of an insulin-like material from spinach and Lemnagibba G3. *Journal of Biological Chemistry*, *262*, 6238–6247. https://doi.org/10.1016/S0021-9258(18)45561-5

De-Wilde, W. J. J. O., & Duyfjies, B. E. E. (2002). Synopsis of *Monordica* (Cucurbitaceae) in SE-Asia and Malaysia. *Botanicheskii Zhurnal. Journal Botanique*, *87*, 132-148.

Fukushima, M., Watanabe, S., & Kushima, K. (1976). Extraction and purification of a substance with luteinizing hormone releasing activity from the leaves of *Avena sativa Tohoku*. *Journal of Experimental Medicine*, *119*, 115–122. https://doi.org/10.1620/tjem.119.115

Gonzalez, G. N. G., Chuc, K. J. A., Torres, C. J. A., Garcia, Z. E. A., Betancur, A. D., Luis, C. G., & Sugey, R. S. G. (2017). Biofunctional properties of bioactive peptide fractions from protein isolates of moringa seed (*Moringa oleifera*). *Journal of Food Science and Technology*, *54*, 4268–4276. https://doi.org/10.1007/s13197-017-2898-8

Jeyadevi, R. A., Sivasudha, T., Rameshkumar, A., Sangeetha, B., Arul Ananth, D., & Smilin B. A. (2012). Nutritional constituents and medicinal values of *Momordica cymbalaria* (*Athalakkai*) - a review. *Asian Pacific Journal of Tropical Biomedicine*, 5456–5461. https://doi.org/10.1016/S2221-1691(12)60206-8

Koneri, R., Balaraman, R., Vinoth, K. M., & Hariprasad. (2007). Cardioprotective effect of *Momordica cymbalaria* fenzl against experimental myocardial injury induced by isoproterenol. *Internet Journal of Pharmacology*, *5*, 2.

Kumar, P., Rao, G. D., Lakshmayya, S., & Ramachandra, S. (2008). Antioxidant and hepatoprotective activity of tubers of *Momordica cymbalaria* Cogn against CCl4 induced liver injury in rats. *Indian Journal of Experimental Biology*, *46*, 510–513.

Kumar, P., Rao, G. D., Lakshmayya, S., & Ramachandra, S. (2011). Nephroprotective and nitric oxide scavenging activity of tubers of *Momordica cymbalaria* in rats Avicenna. *Journal of Medical Biotechnology*, *3*(2), 87–93.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685. https://doi.org/10.1038/227680a0

Leroith, D., Pickens, W., Wilson, G. L., Miller, B., Berelowitz, M., Vinik, A. I., Collier, E., & Cleland, C. F. (1985). Somatostatin-like material is present in flowering plants. *Endocrinology*, *117*, 2093–2097. https://doi.org/10.1210/endo-117-5-2093

Maddirela, D. R., Kameswara, R. B., Kondeti, V. K, Kassetti, R. B., Shaik, S. F., Mekala, T. S. K., & Chippada, A. R. (2010). Isolation and characterization of a novel anti-hyperglycemic protein from the fruits of *Momordica cymbalaria*. *Journal of Ethnopharmacology*, *128*, 58–62. https://doi.org/10.1016/j.jep.2009.12.025

Magdalena, K. C., Dariusz, K., Aneta, L., Aneta, M. P., Rafal, B., & Ewa, G. (2020). Effective callus induction and plant regeneration in callus and protoplast cultures of *Nigella damascene* L. *Plant Cell Tissue & Organ Culture*, *143*, 693–707. https://doi.org/10.1007/s11240-020-01953-9

Mahesh, K. P., Venkataranganna, M. V., Manjunath, K., Viswanatha, G. L., & Ashok, G. (2018). *Momordica cymbalaria* fruit extract attenuates high-fat diet-induced obesity and diabetes in C57BL/6 mice. *Iranian Journal of Basic Medical Sciences*, *21*(10), 1083–1090. https://doi.org/10.22038/IJBMS.2018.29354.7095

Morley, J. E., Meyer, N., Pekary, A. E., Melmed, S., Carlson, H. E., Briggs, J. E., & Hershman, J. M. (1980). A prolactin inhibitory factor with immuno-characteristics similar to thyrotropin releasing factor (TRH) is present in rat pituitary tumors (GH3 and W5), testicular tissue and a plant material alfalfa. *Biochemical and Biophysical Research Communications*, *96*, 47–53. https://doi.org/10.1016/0006-291x(80)91179-1

Munshi, M., Roy, P. K., Kabir, M. H., & Ahmed, G. (2007). *In vitro* regeneration of Cabbage (*Brassica oleraceae* L. var Capitata) through hypocotyl and cotyledon culture. *Plant Tissue Culture & Biotechnology*, *17*(2), 131-136. https://doi.org/10.3329/ptcb.v17i2.3233

Nikam, T. D., Ghane, S. G., Nehul, J. N., & Barmukh, R. B. (2009). Induction of morphogenic callus and multiple shoot regeneration in *Momordica cymbalaria* Fenzl. *Indian Journal of Biotechnology*, *8*, 442-447.

Raghavendra, M., Sushmitha, D., Raghuveer, Y. P., Pradeepkumar, B., Venkatakirankumar, M., & Abbulu, K. (2021). Mitigative effect of *Momordica cymbalaria* fruit extract against sodium fluoride induced hepatotoxicity in Wistar male albino rats. *Journal of Basic and Clinical Physiology and Pharmacology*, *32*(2), 79–87. https://doi.org/10.1515/jbcpp-2019-0362

Rao, B. K., Kesavulu, M., & Apparao, C. (2003). Evaluation of antidiabetic effect of *Momordica cynobalaria* fruit in alloxan diabetic rats. *Fitoterapia*, *74*, 7-13. https://doi.org/10.1016/s0367-326x(02)00297-6

Retamal, C. A., Paula, T., & Elias, W. A. (1999). Protein purification from polyacrylamide gels by sonication extraction. *Analytical Biochemistry*, *268*, 15-20. https://doi.org/10.1006/abio.1998.2977

Robinson, R. W., & Decker-Walters, D. S. (1997). Cucurbits. Cab International, Wallingford, Oxon, New York.

Saravanan, R., Ghouse, B. M., Amzad, B. K., & Vivekanandan, L. (2007). *Ex situ* culture studies on a medicinal plant *Pedalium murex* L. *Indian Journal of Plant Physiology*, *12*(2), 178-180.

Sarmadi, B., Aminuddin, F., Hamid, M., Saari, N., Abdul-Hamid, A., & Ismail, A. (2012). Hypoglycemic efects of cocoa (*Theobroma cacao* L.) autolysates. *Food Chemistry*, *134*, 905–911. https://doi.org/10.1016/j.foodchem.2012.02.202

Schaefer, H., & Renner, S.S. (2010). A three-genome phylogeny of *Momordica* (Cucurbitaceae) suggests sevenretuens from dioecy to monoecy and recent long distance dispersalto. *Molecular Phylogenetics and Evolution*, *l54*(2), 553-560. https://doi.org/10.1016/j.ympev.2009.08.006

Shital, P. P., Ashutosh, G., Kiran, K., & Abhijeet, S. K. (2020). Plant derived bioactive peptides: a treatment to cure diabetes. *International Journal for Peptide Research & Therapeutics*, *26*, 955–968. https://doi.org/10.1007/s10989-019-09899-z

Vaštag, Z., Popovic, L., & Popovic, S. (2014). Bioactivity evaluation of cucurbitin derived enzymatic hydrolysates. *International Journal of Agricultural and Biosystems Engineering*, *8*, 445–448.

Wang, J., Du, K., Fang, L., Liu, C., Min, W., & Liu, J. (2018). Evaluation of the antidiabetic activity of hydrolyzed peptides derived from *Juglans mandshurica* Maxim fruits in insulin-resistant Hep G2 cells and type 2 diabetic mice. *Journal Food Biochemistry*, *42e*, 12518. https://doi.org/10.1111/jfbc.12518

Xianbao, D., Yaqian, X., Jing, L., Dong, Y., Liu, J., Heyun, S., Heng, S. Yunmeng, W., Junyu M., Yanling. L., & Mei, Y. (2020). The establishment of an efficient callus induction system for lotus (*Nelumbo nucifera*). *Plants*, *9*, 1436. https://doi.org/10.3390/plants9111436

(***Received : 20.9.2023; Revised : 25.7.2024; Accepted : 30.7.2024)***