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Determination of antioxidant capacity and flavonoid composition of onion (*Allium cepa* L.) landrace '*Krishnapuram*' bulb using HPLC-ESI-ITMS

G Vijayalakshmi[1], M Muni Raja[1], M Lakshmipathi Naik[1], V Carbone[2], G L Russo[2] and P S Sha Valli Khan[1]*

[1]*Department of Botany, Yogi Vemana University, Vemanapuram, Kadapa, Andhra Pradesh 516003, India*

[2]*Institute of Food Sciences, National Research Council, 83100 Avellino, Italy*

**Corresponding author (Email, pssvkhan@yogivemanauniversity.ac.in)*

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This study reports for the first-time antioxidant activity and flavonoid composition of KP onion landrace which is useful for future breeding programs and to obtain geographical indication (GI) tag for the benefit of farmers. The present study was aimed to determine antioxidant capacity and flavonoid composition of bulbs of red onion (*Allium cepa* L.) landrace '*Krishnapuram*' (KP) from India using high-performance liquid chromatography (HPLC)-Electrospray Ionization (ESI)-multistage Ion Trap Mass Spectrometry (ITMS). The antioxidant activity was assayed by Ferric Reducing Antioxidant Power (FRAP) and hypochlorous acid (HClO)-induced oxidative damage in human erythrocytes. The total phenolic (TPC) contents in KP onion bulb extract (with 80% methanol) found to be 1.10 ± 0.2 mg GAE/g FW and 38.88 ± 1.0 µM QE/g. The FRAP activity measured for the bulb extract was 13.20 ± 0.1 µM QE/g. KP onion bulb extracts protected red blood cells (RBC) effectively (23%) against the oxidative damage induced by HClO. HPLC-ESI-ITMS analysis showed the presence of eight flavonols and five anthocyanins. Quercetin 3,4′-*O*-diglucoside (384.71 ± 0.49 mg/kg FW) and cyanidin 3-(6″-malonylglucoside) (20.95 ± 0.60 mg/kg FW) were detected as major flavonol and anthocyanin, respectively. The study suggests that KP onion has a considerable antioxidant activity due to the presence of high TPC. Moreover, quercetin glucosides are found to be more abundant than quercetin. The differences in quercetin glycosides content among different red onions could be useful for breeding programmes in the future.

Keywords. *Allium cepa*; Antioxidant; Flavonoids; GI tag; HPLC-ESI-ITMS; KP onion

1. Introduction

Allium cepa L. (bulb onion) is cultivated in 175 countries on around 6.6 million hectares (Mha) yielding a production of 742.5 million tons (MT) (FAO 2014). In India, onion was grown on 12.25 Mha with a total production of 20.99 MT and an average yield of 17.1 tons/ha (NHB 2017). The onion bulb is eaten fresh as vegetable, added as essential ingredient in variety of dishes due to its special pungent flavor or for its capacity to spice other foods. In addition, onion bulbs are abundant sources of phenolic compounds, which have high antioxidant activities (AOAs) and other beneficial effects such as decreasing the risk of cancer, cardiovascular, neuro-degenerative and other oxidative stress related disorders (Ren *et al.* 2017). Flavonoids are major sub-classes of phenolics detected in onion bulbs, those are contributing three different colors to the germplasm; red, yellow and white (Donner *et al.* 1997). Two sub-groups of flavonoids such as flavonols and anthocyanins are majorly found in onion bulbs. Flavonols occur in the form of quercetin and its mono

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or diglucosides (Perez-Gregorio *et al.* 2010). Anthocyanins are occurred only in the red onion bulbs, whereas yellow bulbs are usually higher in quercetin (Santas *et al.* 2008). Cultivars, microclimatic conditions, growth stages, methods of cultivation and processing have been found to affect the quality and quantity of flavonoids in onion bulbs and consequently beneficial health effects (Manohar *et al.* 2017).

Antioxidant activities and flavonoid composition have already been reported in different onion landraces from various countries; white onion '*Branco da Povoa'* and red onion '*Vermella da Povoa'* from Portugal (Perez-Gregorio *et al.* 2010), white-brownish onion '*Cipolla di Giarratana'* (Riggi *et al.* 2013), red onion '*Tropea*' (Tedesco *et al.* 2015) and white onion '*Bianca di Pompei*' (Liguori *et al.* 2017) from Italy and red onion '*Vatikiotiko*' from Greece (Petropoulos *et al.* 2015). The onion landrace '*Krishnapuram*', popularly known as 'KP onion' is a short-day and open pollinated cultivar known for producing small size, dark red color and pungent bulbs. This crop cultivated traditionally in the *Krishnapuram* village of YSR Kadapa District, Andhra Pradesh, India (Vijayalakshmi *et al.* 2017). Bulbs are exported to South-East Asian and Middle East countries for consuming fresh in the preparation of salads, sauces and soups. So far there has been no report on the antioxidant activity and flavonoid composition of KP onion. Therefore, the present study is aimed to determine the flavonoid composition and antioxidant capacity of KP onion bulb extract.

2. Materials and methods

2.1 *Preparation of sample*

KP onion bulbs were collected in the month of March 2014 from a farm located in the '*Krishnapuram*' village at (14° 45′ 19″ N and 78° 44′ 44″ E) YSR Kadapa District, Andhra Pradesh, India. The bulbs were transported to the laboratory, then cured and dried in the dark under ambient conditions for two weeks. The outer dry and semi-dry layers of bulbs were removed before chopping into slices. Bulb slices were pulverized in a domestic electric blender. The 10 g of blended bulb material was mixed with 20 ml of (80:20, v/v) methanol/water. The mixture was vortexed for 10 min on a horizontal shaker (VDRL 711, ASAL, Cernuscosul Naviglio, MI, Italy) and then subjected to an ultrasonication bath for 15 min. The onion bulb extract was passed through filter papers (Cordenons, MI, Italy) to remove debris. Filtered onion bulb extract was desiccated in *vacuum* using a rotary evaporator (4000 / HB Efficient model, LaboRota, Heidolph). The resulting dehydrated onion bulb extract powder stored at −20°C for further experiments. Sample preparation was done in duplicate and the exposure to light was prevented.

2.2 *Determination of total phenolic content (TPC)*

The methanolic onion bulb extract powder was suspended in phosphate-buffered saline (PBS) at 50 mg/ml concentration and the stocks were kept at 4°C temperature in the dark. The total phenol content of onion bulb extract was determined by using Folin-Ciocalteu's Reagent (FCR) (Singleton *et al.* 1999) with slight modifications (Tedesco *et al.* 2015). Onion bulb extract was mixed with FCR and the mixture was incubated in the dark at room temperature for 30 min. Subsequently the reaction was neutralized with 7.5% (w/v) sodium carbonate in water. The optical density (OD) of resulting blue solution was calculated at 760 nm using spectrophotometer (DV730, Beckman Coulter, Brea, CA, USA) against a blank, consisting of distilled water and reagent. The total phenolic content was calculated from the linear equation of a standard curve prepared with either gallic acid (GAE) or quercetin (QE). The phenolic content was expressed as mg of GAE equivalents per 100 g fresh weight (mg GAE/g FW) or QE equivalents per 100 g fresh weight (mg QE/g FW).

2.3 *Determination of ferric reducing antioxidant power (FRAP)*

Ferric reducing antioxidant power (FRAP) activity was measured as per Firuzi *et al.* (2005) with slight modifications (Tedesco *et al.* 2015). FRAP solution was prepared by combining ferric chloride (20 mM) and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 300 mM acetate buffer (at pH 3.5). The sample (onion bulb extract powder) and FRAP solution were mixed and kept at room temperature for 30 min. Thereafter, OD values were taken at 595 nm using spectrophotometer (DV730, Beckman Coulter, Brea, CA, USA) against the blank to determine the reducing power. Calibration was carried out using quercetin (QE) and values expressed as micromolar (μM) QE equivalents per milligram (mg) per ml of onion extract.

2.4 *Hypochlorous acid-induced oxidative damage of human erythrocytes*

The erythrocytes (RBC) collected from healthy consenting donors were rinsed twice with cold PBS. Then erythrocytes were re-suspended in PBS (1.2×10^5 cells/μl). Sodium hypochlorite (NaOCl) was dissolved in PBS. The pH of the solution was adjusted to 7.4 just prior to use. At this pH, the solution comprises HClO and OCl^- at almost equimolar ratio and then stated to as HClO (Visssers *et al.* 1998) The OCl^- concentration was estimated referring an absorbance coefficient of 350 mol^{-1} cm^{-1} at 292 nm by spectrophotometer. The erythrocytes samples were pre-treated with KP onion extract at 0.25 mg/ml concentration for 45 min and followed by addition of 0.3 mM of HClO. The incubation was carried at 37°C temperature for 15 min. After incubation, RBC suspensions (untreated, treated with 0.25 mg/ml onion extract, 0.3 mM HClO and onion extract and HClO) were centrifuged at 2000g for 2 min. The vulnerability of erythrocytes to HClO led to oxidative damage was determined in terms of the percent of haemolysis at 540 nm using spectrophotometry (Tedesco *et al.* 2015).

2.5 *HPLC quantitative analysis*

KP onion bulb extract powder was suspended in 1% (v/v) formic acid. HPLC analysis was done using Agilent 1110 Series HPLC system (Agilent, Palo Alto, CA, USA) consisting of a binary pump (G-1312A) and a UV detector (G-1314A). The separation was done with a Hypersil BDS C18 column (250 mm x 4.6 mm, 5 μM particle size, Thermo, Bellefonte, PA, USA). The mobile phase comprised of solvent A and B. Solvent A consists of 1% formic acid in water, whereas solvent B comprises 1% (v/v) formic acid in methanol: water (50:50, v/v), respectively. The elution program was as follows; step I 0.0–5.0 min (hold with 20% solvent B), step II 5.0–27.0 min, elution from 20% (B) to 80% (B), step III 27–40 min, isocratic elution at 80% B, step IV 40.0–50.0 min from 80% (B) to 95% (B), step V 50–65 min maintenance at 95% B. The flow rate of the sample was continued at 1.0 ml/min. The volume of the injector was selected at 100 μl. Absorbance of eluents was measured at 340 nm for flavonols and 520 nm for anthocyanins.

Reference curves for each flavonol standard (quercetin 3-*O*-glucoside, quercetin 3,4′-di-*O*-glucoside, quercetin 4′-*O*-glucoside and quercetin) were prepared in six concentrations in the range from 1 to 180 μg/ml. Anthocyanin quantification was done by using external calibration curves made by repetitive injections of cyanidin 3-*O*-glucoside in five concentrations in the range of 0.5–20 μg/ml. The duplicate injections at each level were did for flavonols and anthocyanins. All the samples were prepared and analysed two times. TPC (total phenolic content) was calculated as the sum of values of the individual phenolic compounds. The values were conveyed as milligrams (mg) per kilogram (kg) fresh weight (FW).

2.6 *ESI-ITMS^n analyses*

The HPLC separated fractions were subjected to ESI (Electrospray Ionization)- multistage ITMS (Ion Trap Mass Spectrometry^n) using a Finnigan LCQ DECA XP Max ion trap mass spectrometer (Thermo Finnigan, CA, USA) for the detection of phenolic compounds. The ITM was equipped with *X calibur* system manager data acquisition software. MS analyses were followed as described by Tedesco *et al.* (2015). Mass spectra were documented from *m*/*z* (mass-to-charge ratio) 50 to 1500 both in positive and negative ionization modes. The data were obtained in MS, MS/MS and MS^n scanning modes.

2.7 *Statistical analysis*

All experiments were repeated twice, and the resulting data were expressed as mean ± standard deviation (SD). In HPLC quantitative analysis, each replicate solution was injected two times for uniform analysis and identification of compounds.

3. Results

3.1 *Total phenolic content and FRAP*

The total phenolic content in KP onion bulb (extracted with 80% methanol and by ultrasonication) was found to be 1.10 ± 0.2 mg GAE/g FW extract. In terms of quercetin, total phenolic content in KP onion bulbs was also calculated to be 38.88 ± 1.0 μM QE/g FW. The FRAP activity measured for KP onion bulb extract was found to be 13.20 ± 0.1 μM QE/g.

3.2 *HClO induced oxidative damage*

RBCs treated with 0.3 mM HClO resulted in about 6% of hemolysis (figure 1). In contrast, an addition of KP

onion extract at 0.25 mg/l (w/v) concentration to RBCs for 45 min resulted in a protective effect in reducing HClO-induced hemolysis (23%), although the difference between the two time-points in figure 1, i.e. HClO *vs* KP onion + HClO, is at the edge of statistical significance ($P = 0.075$). The applied concentration (e.g., 0.25 mg/ml) to RBCs was not hemolytic *per se.*

3.3 *Identification of flavonoids in the onion extract*

The HPLC chromatogram extract of KP onion bulb showed 8 peaks (1 to 8 peaks; supplementary figure 1A) and five (9 to 13 peaks; supplementary figure 1B) for spectral properties of flavonols and anthocyanins, respectively with different retention times between 16 min to 40 min (table 1). The eight flavonols present in the KP onion bulb were identified by ESI-ITMS^n as quercetin-3,7,4′-triglucoside (peak 1), quercetin-7,4′-diglucoside (peak 2), quercetin-3,4′-diglucoside (peak 3), isorhamnetin-3,4′-diglucoside (peak 4), quercetin-3-*O*-glucoside (peak 5), quercetin-4-*O*-glucoside (peak 6), isorhamnetin-4′-glucoside (peak 7) and quercetin (peak 8) (supplementary figure 1A; table 1). A total flavonol content found to be 827.02 ± 3.94 mg/Kg FW (table 2). The polar extract contains mainly quercetin-3, 4′-*O*-diglucoside (384.71 ± 0.49 mg/kg FW) (table 2). Quercetin-3, 4′-*O*-diglucoside and quercetin-4-*O*-monoglucoside represent over 46–47% and 34–35% of the total fraction, respectively (table 2). The two major flavonols quercetin 3,4′-diglucoside and quercetin 4′-glucoside, represented 85% and 95% of the total flavonols. Quercetin

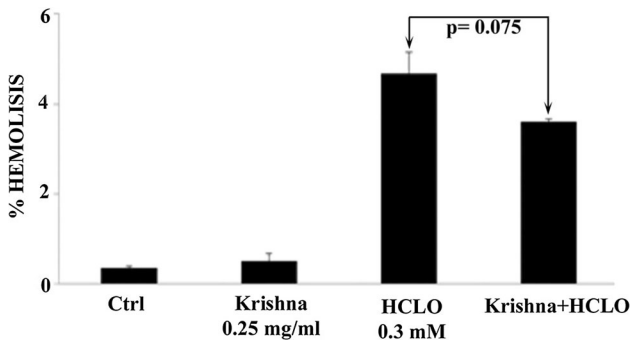

Figure 1. Effect of KP onion extract on hemolysis of human erythrocytes; RBCs from 2 healthy donors (in duplicate) were stimulated with 0.25 mg/ml extracts prepared from KP onion and incubated for 45 min at 37°C; samples also prepared with or without 0.3 mM HClO for 15 min at 37°C. Hemolysis was expressed in terms of the percentage (%). Bar graphs represent the mean ± SD.

represented as minor component or even below detection limits in KP onion.

The five peaks present in the HPLC chromatograms were identified as cyanidin 3-glucoside (t_R = 24.70 min) (peak 9); cyanidin 3-laminaribioside (cyanidin 3-glucosylglucoside) (t_R = 25.89 min) (peak 10); cyanidin 3-(3″-malonylglucoside) (t_R = 26.81 min) (peak 11); cyanidin 3-(6″-malonylglucoside) (t_R = 30.67 min) (peak 12) and cyanidin 3-malonyl-laminaribioside (cyanidin 3-malonylglucosylglucoside) (t_R = 31.85 min) (peak 13) (supplementary figure 1B; table 1). The total content of anthocyanins in KP onion was found to be 36.7 ± 1.04 mg/kg FW, which is higher than that measured in other red onion varieties. Quantitative analysis showed that cyanidin 3-(6″-malonylglucoside) is the most abundant anthocyanin (20.95 ± 0.60 mg/kg FW) followed by cyanidin 3-malonyl-laminaribioside (7.92 ± 0.25 mg/kg FW), cyanidin 3-glucoside (3.79 ± 0.06 mg/kg FW), cyanidin 3-laminaribioside (3.41 ± 0.06 mg/kg FW) and cyanidin 3-(-3″-malonylglucoside) (0.64 ± 0.01 mg/kg FW) (table 2). The content of cyanidin 3-glucoside in KP onion was found to be 3.79 ± 0.06 mg/kg FW.

4. Discussion

Total phenolics (TPC) from KP onion bulbs were extracted with 80% methanol by subjecting to ultrasonication. Solvents such as methanol, ethanol, acetone and glycerol and their aqueous mixtures were routinely used for the extraction of phenols and flavonoids from onion bulbs. However, the content of TPC of onion depended on the type, polarity and purity of the solvent used for extraction. Low yields of phenols were obtained with pure solvent as compared to aqueous-organic-solvent (Lu *et al.* 2011). Santas *et al.* (2008) used various solvent compositions and suggested methanol (80%) as the best solvent for obtaining higher content of phenolics as well as flavonoids from onion bulbs. The present study agrees with the results of Santas *et al.* (2008) and used 80% methanol for the extraction of polyphenols from KP onion bulbs. Ultrasonication can facilitate extraction processes both by cell disruption and by providing a suitable dissipation of energy for proficient mass transfer in the boundary layer surrounding the sold matrix, hence aid release of polyphenols (Vetal *et al.* 2013). For HPLC quantitative analysis, formic acid (1%) was incorporated with the solvent methanol for the separation of flavonoids present in the KP onion bulb to lower the

Table 1. List of compounds identified in KP onion extract by HPLC-UV/Vis and ESI-ITMS^n(as per supplementary figure 1)

Peak	t_R (min)	$[M-H]^-$ *m/z*	MS/MS ions *m/z*	Identification
1	16.99	787	625,463,301	Quercetin-3,7,4′-triglucoside
2	23.26	625	463,301	Quercetin-7,4′-diglucoside
3	25.53	625	463,301	Quercetin-3,4′-diglucoside
4	26.90	639	477,315	Isorhamnetin-3,4′-diglucoside
5	29.89	463	300	Quercetin-3-*O*-glucoside (Isoquercitrin)
6	32.21	463	301	Quercetin-4-*O*-glucoside
7	35.53	477	315	Isorhamnetin-4′-glucoside
8	40.81	301	179,151	Quercetin
9	24.70	449	287	Cyanidin 3-glucoside
10	25.89	611	449,287	Cyanidin-3-laminaribioside (Cyanidin 3-glucosylglucoside)
11	26.81	535	449,287	Cyanidin 3-(3′′-malonylglucoside)
12	30.67	535	491,449,287	Cyanidin 3-(6′′-malonylglucoside)
13	31.85	697	535,449,287	Cyanidin-3-malonyl-laminaribioside (Cyanidin3-malonylglucosylglucoside)

Table 2. Concentration of individual and total polyphenolics determined by HPLC (mg/Kg of FW) extracted from KP onion bulbs

	Mean concentration ± SD
Flavonols	
Quercetin-3,7,4′-triglucoside	6.29 ± 0.27
Quercetin-7,4′-diglucoside	5.14 ± 0.22
Quercetina-3,4′-diglucoside	384.71 ± 0.49
Isorhamnetin-3,4′-diglucoside	37.09 ± 0.80
Quercetin-3-*O*-glucoside (Isoquercitrin)	20.62 ± 0.73
Quercetin-4-*O*-glucoside	282.03 ± 0.48
Isorhamnetin-4′-glucoside	87.44 ± 1.85
Quercetin	3.68 ± 0.07
Total flavonols	827.02 ± 3.94
Anthocyanins	
Cyanidin 3-glucoside	3.79 ± 0.06
Cyanidin 3-laminaribioside (Cyanidin 3-glucosylglucoside)	3.41 ± 0.15
Cyanidin 3-(3′′-malonylglucoside)	0.64 ± 0.01
Cyanidin 3-(6′′-malonylglucoside)	20.95 ± 0.60
Cyanidin 3-malonyl-laminaribioside (Cyanidin 3-malonylglucosylglucoside)	7.92 ± 0.25
Total anthocyanins	36.72 ± 1.04
Total polyphenols (by HPLC method)	863.74 ± 2.89

Results were expressed as average (mean) concentration ± SD of duplicates.

pH and to keep the anthocyanins in their stable flavillum form (Perez-Gregorio *et al.* 2010).

Total phenolic content of KP onion bulb extracts was estimated by FCR assay and expressed in terms of either GAE or QE. The GAE of KP onion was reported to 1.10 ± 0.2 mg GAE/g extract. The GAE content was varied in red onion bulbs from various countries (Lu *et al.* 2011; Petropoulos *et al.* 2015). Total phenolic content in KP onion bulbs was also calculated to be 38.88 ± 1.0 μM QE/g FW, which is higher as compared to onions from Italy, e.g. '*Tropea*' red onion (29.35 ± 0.03 μM QE/g FW) and *Montoro* copper onion (12.68 ± 0.02 μM QE/g FW) (Tedesco *et al.* 2015) (supplementary table 1). The present study also indicated the more abundance of quercetin in KP onion than gallic acid. The composition and content of phenolics varied in onion bulbs due to genetic differences and different agricultural practices, environmental factors, maturity, harvest season and even post-harvest practices (Ren *et al.* 2017; Liguori *et al.* 2017).

Antioxidant activity of onion bulbs is depended on phenolics and other bioactive compounds (e.g. organosulfur compounds, allicin). Sharma *et al.* (2013) reported that phenolics especially flavonoids contribute to antioxidant capacity more than bioactive compounds. Two are more methods are always suggested for the assessment of AOA in vegetables (Chu *et al.* 2000). The present study employed two different methods for assessing AOA in KP onion; (1) FRAP for detection of reducing power and (2) hypochlorous acid-induced oxidative damage of human erythrocytes for assessing ROS scavenging activity. FRAP causes reduction of TPTZ-ferric (Fe^{3+}) complex to TPTZ-ferrous (Fe^{2+}) complex by an antioxidant at low pH (Benzie and Strain 1996). FRAP activity measured for KP onion bulb extract was 13.20 ± 0.1 μM QE/ g, which was higher than the activity of '*Tropea*' red

onion (4.79 ± 0.02 μM QE/g) and *Montoro*' copper onion (1.67 ± 0.01 μM QE/g) (Tedesco *et al.* 2015). Further, FRAP assay showed that the red onion varieties had the highest AOA than other types of onions (supplementary table 1).

RBCs remarkably control intracellular oxidative stress. The treatment with HOCl to RBCs causes lysis of RBC cells and burst of intracellular ROS. Pretreatment of whole blood cells with onion extracts generates a protective effect against HClO-induced hemolysis (Tedesco *et al.* 2015). Experimental data reported in figure 1 agrees with previous studies by Tedesco *et al.* (2015) that '*Tropea*' and '*Montoro*' onion extracts indicating their ability to counteract the HOCl induced damages on RBC under similar experimental conditions. However, in comparison, extracts from these two onions protected RBCs more effectively (30–40%) than KP alone (23%), although the latter extract is high in both polyphenolic content and antioxidant activity.

The HPLC chromatogram of KP onion bulb extracts showed the occurrence of flavonols as quercetin and its glucoside derivatives. These observations were similar to the findings of previous reports on onion (Tedesco *et al.* 2015; Kwak *et al.* 2017). Quercetin represented as minor component than its glucosides in KP onion. In fact, quercetin is generally reported in literature to exist in high content in red onions ranging from 54 to 286 mg/kg FW (Patil *et al.* 1995). Five types of cyanidin anthocyanins were detected in KP onion. These results agree with the findings of Donner *et al.* (1997) and Tedesco *et al.* (2015). The total content of anthocyanins in KP onion was found to be 36.7 ± 1.04 mg/kg FW, which is higher than that measured in other red onion landrace such as *Vermellha da Povoa* (28.6 mg/kg FW) (Peres-Gregorio *et al.* 2010). The content of cyanidin 3-glucoside in KP onion was found to be 3.79 ± 0.06 mg/kg FW which is higher than that in *Vermellha da Povoa* (1.6 mg/kg FW) (Peres-Gregorio *et al.* 2010) and *Montoro* ecotype (1.19 mg/kg FW) (Tedesco *et al.* 2015).

5. Conclusion

In conclusion, this study reports for the first-time antioxidant capacity and analysis of flavonoid composition of whole bulbs of KP onion. This information about quality attributes is useful for the conservation and future breeding programmes for the improvement of this onion landrace. The chemical characterization is also the first step in the exploitation of such agriculturally unique product to obtain geographical indication (GI) tag for the benefit of the farmers in India. The genetic characteristics, agronomic practices and the climate in the region of cultivation are determining the flavonoid contents in KP onion.

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