IN VITRO ANTIOXIDANT EVALUATION OF HYDROALCOHOLIC AND ORGANIC SOLVENT EXTRACTS OF COMMON KASHMIRI *HAAKH* (LOCAL COLLARD GREENS)

Shabir Ahmad Mir[1]
Division of Veterinary Pharmacology & Toxicology,
Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir,
F.V.Sc. & A.H., Shuhama, Srinagar-190001, J & K, India
Zari Mohalla, Lal Bazar, Srinagar, Kashmir (India)
Formerly Professor & Head, SKUAST-K; superannuated April 1st 2014

ABSTRACT: The main objective of the investigation has been to evaluate the extracts of common Kashmiri *Haakh* (local collard greens, identified as *Brassica olerecea* var Haka Javied) for antioxidant activities. A battery of locally improvised *in vitro* inexpensive methods have been employed to confirm the antioxidant potential of the vegetable most widely consumed in the Kashmir valley. The activity of the extracts has ranged from marked reduction towards ions of permanganate, iodine, iron (III), dichromate and MTT to remarkable scavenging activity towards reactive oxygen species including peroxide ions, hydroxyl radicals (Fenton reaction), nitrous acid / nitric oxide and superoxide anions generated from alkaline pyrogallol and alkaline hydroxylamine. Phytochemical analysis has revealed presence of phenolics and flavonoids in alkaline hydroalcoholic extract, beta-carotene in chloroform-petroleum ether extract and thiocyanate in water-soluble extract obtained from organic solvent soluble extract. The work confirms obvious potential advantages of the vegetable for the local population.

Keywords: Local collard greens, common Kashmiri *Haakh*, *Brassica olerecea* var Haka, antioxidant activity, reactive oxygen species, radical scavenging activity

INTRODUCTION

Collard greens (*Brassica oleracea* var viridis) is a leafy vegetable that is nutritionally rich, most popular and widely consumed in different parts of the world (Nierenberg, 2012; Wikipedia, 2017; Mateljan, 2018). Common Kashmiri *Haakh* is an indigenous cultivar of collard greens which has been recognized locally as *Brassica oleracea* var Haka Javied (Kak, 1983). It is the most common leafy vegetable in the Kashmir valley and consumed extensively as a staple diet in a variety of forms and recipes.

The term *Haakh* is loosely used in the valley to refer to a variety of different leafy vegetables belonging to different plant families including *waste Haakh* (*Atriplex hortensis*; Chenopodiacea), *wappal Haakh* (*Dipsacus inermis*; Caprifoliacea) and *pumb Haakh* (*Rheum emodi*; Polygonacea). Only Common Kashmiri *Haakh* (cultivar group Acephala) and *munje Haakh* (cultivar group Gongylodes, identified as *Brassica oleracea* var caulorappa; kohlrabi, knolkhol) share the common genus, *Brassica oleracea* and common family, Brassicacea.

Collard greens are popularly recognized as one of the world's healthiest foods. They are a rich reservoir of nutrients including a plenty of antioxidants ranging from minerals (Zn, Mn, Se), vitamins (C, E), carotenoids (beta-carotene, alpha-carotene, lycopene, lutein, zeaxanthin, cryptoxanthin), phenolics (caffeic and ferulic acids), flavonoids (quercetin and kaemferol), alpha-lipoic acid (Ware, 2016; Adams, 2018), the latter with recognized biological antioxidant activity (Packer et al., 1995) and glucosinolates providing potential bioactive isothiocyanates, sulforaphane, indole-3-carbinol and its dimer di-indolylmethane (DIM) (USDA, 2008; Nierenberg, 2012; Tang et al., 2013; Lixandru, 2015; Mateljan, 2018; Rudraappa, 2018).

Antioxidant potential of collard greens is recognized primarily due to its phytochemical constituents with known antioxidant potentials. There are limited actual antioxidant studies having been done on collard green extracts. These too are limited to their free radical scavenging activity (RSA) using DPPH assay (Melo et al., 2006; Clifford & Dawson, 2012) and beta-carotene bleaching following linoleic acid peroxidation (Melo et al., 2006) or with isolated indole-3-carbinol, identified in crucifers, showing strong free radical scavenging activity (Arnao et al., 2008). Similarly, antioxidant activity has been shown in a closely related cultivar of collard greens such as kale with demonstratable RSA based on DPPH assay (Ligor et al., 2013; Agarwal et al., 2017)· lipid peroxidation in beta-carotene/linoleic acid assay(Ismail et al., 2004), RSA using ABTS substrate (TROLOX equivalents) (Silkora & Bodziarczyk, 2012), and ferric iron reducing activity assay (FRAP) recommended for foodstuffs in general including kale (Halvorsen et al., 2002) and for some Kashmiri wild genotypes with suffix *Haakh* (FRAP) (Murtaza et al., 2005).

In view of wider popularity of Common Kashmiri *Haakh* in local diet and of very limited availability of direct antioxidant studies on the vegetable, present work was mooted to undertake various in vitro antioxidant studies on hydroalcoholic and lipid soluble extracts obtained from freshly collected vegetable leaves employing a battery of inexpensive antioxidant assays improvised in the laboratory.

MATERIALS AND METHODS

The experiments were carried out at an ambient temperature of 13.5 ± 0.5 ^{0}C (range 7 to 17 ^{0}C). The drugs and chemicals used were of standard purity and quality obtained from reputed sources in India. Spectrophtometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan).

Chemicals and reagents

Ammonia solution: Ammonia solution was approximately made as 1 M solution in water from stock ammonia solution.

Sodium hydroxide solution: 1 M, 0.2 M, 0.1 M in water

Sodium bicarbonate solution: 4.2 % (w/v) sodium hydrogen carbonate in water.

Sodium carbonate solution: 1 M sodium carbonate in water.

Potassium ferrocyanide solution: 1.5 % (w/v) potassium ferrocyanide in 1% (w/v) sodium carbonate solution in water.

Folin-Ciocalteu reagent: 2 N

Ferric chloride solution: 1 µmole per mL water.

Lime water: 10 g Ca (OH) $_2$ added to 1 L water; shaken well; left overnight; clear supernatant used for extraction with estimated alkalinity about 0.04N.

Ferric ammonium sulfate solution: Ferric ammonium sulfate solution was prepared by dissolving 500 mg salt in water added 1.7 mL 1% HCl in a total volume of 53.4 mL to provide estimated 20 µmole iron (III) per about 0.005 N HCl.

Dilute HCl : 10% (v/v) concentrated HCl in water.

Dilute H_2SO_4: 10% (v/v) concentrated sulfuric acid in water

Gallic acid solution: 100 µg gallic acid per mL water containing 1% ethanol prepared from stock solution 0.05% (w/v) in 5 %(v/v) ethanol in water

Potassium permanganate solution: 1 µmole $KMNO_4$ per mL water.

Iodine solution: 5 µmole iodine per mL water.

Hydrogen peroxide solution: Working solution made freshly as 2 µmole per mL water for aqueous extracts and as 2.5 µmole H_2O_2 mL^{-1} methanol for organic solvent extracts.

Potassium iodide solution: Potassium iodide as 10% either in water or in methanol (w/v) with overall KOH in stock as 0.002 M as stabilizer.

Sodium bicarbonate solution: $NaHCO_3$ solution 4.2% (w/v) in water.

Sodium chloride solution: 1 M NaCl made in water.

Potassium thiocyanate solution: 10% (w/v) potassium thiocyanate in water.

Beta-carotene solution: 0.1 % (w/v) beta-carotene (Laboratory standard) (Mir et al, 2014) made in chloroform as stock. Diluted solutions made in chloroform as per need.

Sulfanilamide solution: 1% (w/v) sulfanilamide in 1% HCl in water.

Hydrochloric acid: The strength was titrimetrically evaluated as approximately 11.7 M.

MTT reagent: MTT dissolved in methanol to provide 0.1 % (w/v) solution. Each mL provides 100 µg MTT equivalent to 241 nmole MTT.
NEDA solution: 1% and 0.2 % (w/v) NEDA in 1% (v/v) HCl in water.
Nitrite solutions: Appropriate strengths of nitrite solution were made by dilution in water from stock sodium nitrite solution (0.15% (w/v) in water containing 0.2 % (v/v) chloroform in water). For nitrite analysis, dilution was made to provide 10 µg nitrite per mL (0.1 mL stock made 10 mL with water), and for nitrous acid scavenging activity, dilutions were made to provide 0.5 µmole nitrite mL^{-1} water (0.5 mL stock made 21.7 mL with water).
Potassium dichromate solution: 10 µmole as dichromate per mL water as working standard.
Iron-catechol reagent: The reagent was prepared freshly on the day of use by mixing stock solutions of catechol (1% (w/v) in water with about 0.012 M HCl) and ferrous ammonium sulfate (FAS-II) (25 µmole mL^{-1} in 0.005 M HCl), respectively as 2.5 and 1 mL in 50 mL water to provide 0.5 mg catechol and 0.5 µmole of FAS-II per mL reagent.
Pyrogallol solution: Stock pyrogallol solution was made in water (100 mg pyrogallol added to 3.4 mL 0.1 % HCl and 16.4 mL water to provide 40 µmole pyrogallol mL^{-1} about 0.002 M HCl in water. Working pyrogallol solution was made by mixing 0.4 mL stock to 15.6 mL water for water soluble extract assays or methanol for chloroform soluble extract assay to provide 1 µmole pyrogallol mL^{-1} 97.5% methanol
Hydroxylamine solution: The solution was prepared as 0.7 % (w/v) hydroxylamine hydrochloride in water providing 100 µmoles per mL water**.** Working solutions were prepared as per need by dilution with water.
Guaiacol solution: The solution was prepared as 1% (v/v) guaiacol (labeled density, 1.12-1.13 g mL^{-1}) in 20% (v/v) ethanol in water.
SNP solution: Working solutions of sodium nitroprusside were prepared in water from stock solution 0.3% (w/v) in water (equivalent to 10 µmole SNP mL^{-1} water).
Rutin trihydrate: The requisite dilutions were made from stock solution made in methanol providing 2.0 µmole of the flavonoid mL^{-1}methanol.

Preparation of sample extracts

The *Haakh* variety employed throughout the study has been '*aaram Haakh*' (a.k.a.'*buod Haakh*', wherein *buod* in Kashmiri language means 'bundled' so-called because of its availability in the market as bundled leaves). The samples were freshly procured from local market, freed off adhering visible impurities, washed with distilled water, blotted dry, homogenized into a pulpy mass using an electric blender. The homogenate was processed for preparation of various extracts viz., organic phase soluble extract and alkaline hydroalcoholic soluble extract.

Organic phase extract (OPE)

Fifty gram homogenate was added 4 volumes of organic solvent mixture (3:1) chloroform and petroleum ether. Extraction was allowed in dark overnight, and then filtered vide WM Filter No.1. The filtrate was carefully dried to residue with continuous swirling over hot water (50 to 60 ^{0}C) while adding few mL ethanol towards the end. The oily residue on cooling to room temperature was reconstituted in chloroform. Each mL of the extract represented 2.3 g of original vegetable material.
An aliquot of chloroform extract was dried to residue over hot-water bath, the residue was added water equal to the original volume of the aliquot, and the aqueous extract was obtained by filtration over Whatman Filter No.1. The extract called thiocyanate extract (TE) was assayed for chloroform and water soluble thiocyanate content.

Alkaline phase hydroalcoholic extract (APE)

The marc obtained as above was rinsed by percolation with petroleum ether till effluent was almost colorless. The marc was dried over hot water to remove organic solvent residue, air-dried to a free flowing powder. A measured aliquot of powder was extracted with addition of calcium hydroxide (100 mg per g original homogenate) and lime water (5 mL g^{-1}original homogenate) as usual overnight , and filtrate collected vide WM Filter No.1 . The filtrate was added ethanol to provide an overall 70 % ethanol concentration, and then re-filtered. Alkaline alcoholic filtrate in measured volume was reduced to 1/4th of its volume on hot sand-bath to eliminate its ethanol content. The residual extract was appropriately diluted with water. Each mL extract represented 300 mg of original plant material.

Analytical techniques using chemical assays

Phenolic assay: Phenolic content in the extracts was measured by using a modification of Folin-Ciocalteau method (Pourmorad et al., 2006; John et al., 2014). Four milliliter water without and with test extract were each added 0.2 mL of Folin-Ciocalteau reagent (2N) and 0.8 mL water. The mixture was added 3 mL 1M sodium carbonate solution, well mixed up and allowed standing at room temperature for 15 to 20 minutes, and monitored at 520 nm. Standard calibration samples employed 0, 20 through 80 µg of gallic acid in 4 mL of matched solvent.

Flavonoid assay: Standard HCl method (Mir et al., 2013a, 2014a) has been employed to estimate flavonoid content in the extracts. Standard samples contained rutin 0, 20 through 100 µg in matched solvent in 1 mL. Test samples included 125 mg equivalent homogenate extract in 1 mL matching solvent. Each sample was added 3 mL concentrated HC. The samples were allowed standing for 30 to 40 minutes at room temperature, and then monitored spectrophotometrically at 405 nm against reagent blank.

Thiocyanate assay: The method employed for determination of thiocyanate was a modification of recommended procedure (Basova et al., 2014). The extract employed was aqueous extract obtained from chloroform soluble homogenate residue (TE). Two mL aliquot of water contained TE equivalent to *Haakh* homogenate 240 through 800 mg with corresponding controls. Standard samples contained 2 mL water with 0, 20 through 100 µg SCN. Each sample was added 1 mL ferric ammonium sulfate solution (20 µmole iron (III)), allowed standing at room temperature for 10-15 minutes, and monitored at 480 nm. Sample extracts with corresponding masses of extracts in 2 mL water were added 1 mL water instead of iron to serve as corresponding controls.

Beta-carotene assay: Beta-carotene equivalent activity of organic phase extract was determined by comparing absorbance of diluted OPE, containing homogenate equivalent 16 to 48 mg, in chloroform with respect to standard beta-carotene solution made in chloroform over test range 0, 6 through 24 µg in a total 4 mL volume showing maximum absorbance in chloroform at 465 nm (Simpson et al., 1985).

General antioxidant assay methods

Permanganate reducing activity: One mL water containing 1µmole of $KMNO_4$ was added 2 mL water containing varying masses of the extract and 1 mL 20% H_2SO_4. Standard calibration assay was run in absence of extract with 0, and 0.1 through 1µmole of $KMNO_4$ under identical test conditions. The samples were monitored at 540 nm following 15 minutes standing at room temperature. Extract treated samples developing opalescence were centrifuged and supernatant read at 540 nm. The technique details and its scope for various antioxidants have been already published (Mir, 2016a).

Iodine reducing potential (Iodimetric): Two mL water contained appropriate mass of the extract and 3 µmole of iodine. Standard samples contained 0, 0.3 through 5 µmole of iodine in 2 mL water. Each was added 0.5 mL 10% HCl and 1.5 mL water; allowed standing at room temperature for 15 to 20 minutes, and then monitored at 450 nm. The details of the technique and its suitability have been given elsewhere (Mir, 2016b).

Dichromate reducing activity: The method and its applications have been described elsewhere (Mir, 2015a). Standard samples contained 3.5 mL water containing potassium dichromate 0, 0.5 through 3 µmole while test samples contained 3.5 mL water with 100 through 300 mg extract, each with 3 µmole dichromate. Each sample was added 0.5 mL 20% concentrated sulfuric acid. The samples were allowed standing at room temperature for about an hour and then read at 450 nm.

Ferric iron reducing activity (thiocyanate method): Standard methods applicable to water soluble and chloroform based extracts and their scope with respect to alpha-tocopherol and beta-carotene have been given in detail elsewhere employing thiocyanate and salicylate methods for assessing reduction in ferric iron (Mir et al., 2014b).

For assaying APE, three mL aliquot containing 0 and 0.1 through 0.5 µmole ferric iron in water as calibration standard, and test samples containing 0.5 µmole iron with 50 mg homogenate extract. The samples allowed standing for 10 minute and then added 0.5 mL 10% HCl and 0.5 mL 10 % KSCN in that order. The color monitored at 480 nm after 15 to 20 minute standing.

For assaying OPE, one mL chloroform with or without test extract in chloroform is added 1 mL ferric iron 5 µmole in methanol. Standard samples contained 0, 0.6 through 3 µmole $FeCl_3$ under identical conditions. The samples were well-mixed, stoppered and allowed to stand at room temperature for 30 to 40 minutes, and then each sample was added 5 mL 1% sodium chloride to facilitate easy separation of phases on centrifugation. One mL supernatant was added 1 mL 1% HCl followed by 0.5 mL 10% KSCN and 1.5

mL water; allowed standing for 15 to 20 minutes, and monitored at 480 nm for reduction in ferric iron in terms of calibration standards.

MTT reduction activity: The method is a modified version of the technique (Madesh & Balasubramanian, 1998) based on formazan formation with absorption extinction coefficient 17000. Two mL water contained 0, 50 through 150 mg homogenate extract (pH adjusted to about 7.0 for the extract). Each sample was added 1 mL methanol containing 100 µg MTT followed by 0.5 mL 5 M NaOH solution; allowed standing in incubator at 25 ^{0}C for 30 minutes. Each sample was added 4 mL formamide to dissolve the formazan precipitate; and read at 540 nm.

Calculation: Formazan produced in the sample was estimated by using the following formula employing molar extinction coefficient for formazan as 17000:

Formazan produced (or MTT reduced) in µmole g^{-1} homogenate was computed as under: $[A_s * 10^6] / [C_s * 17000] = [A_s * 10^3] / [C_s * 17]$

A_s is absorbance value reaction mixture with test sample

C_s is concentration of sample in the reaction mixture (mg mL^{-1})

Special antioxidant assays

Peroxide scavenging potential (iodometric): An iodometric assay described for copper reduction (Mir, 2015a) has been adapted to monitor peroxide scavenging potential of APE and OPE. For OPE assay, 1mL methanol with appropriate mass of hydrogen peroxide (blank, 0 µmole), standards (0.5 through 2.5 µmole) and samples (2.5 µmole) was added 0.5 mL chloroform without and with test extract wherever required. Each sample was added 0.5 mL 10% HCl followed by 0.1 mL $NaHCO_3$ solution till degassing ceased, then each sample was added 1 mL KI solution, allowed 30 minute standing followed by addition of 2 mL water followed by gentle-shaking and centrifugation. The supernatants were monitored at 450 nm for iodine release in terms of calibration standard. The assay for APE was identical to the followed protocol except all test samples, standard and reagents were prepared in water.

Hydroxyl radical scavenging activity (Fenton catechol method) : Standard samples contained 1 mL iron-catechol reagent with 2 mL water containing 0, 0.25 through 2.5 µmole of H_2O_2. Test samples contained 1 mL iron-catechol reagent with appropriate mass of extract in absence and presence of standard 2.5 µmole of H_2O_2 in 2 mL water. The samples were allowed standing at room temperature for 20 to 30 minutes to complete oxidation, and then each sample was added 1 mL water, and the absorbance was monitored at 360 nm within next 10 minutes. The method and its scope in detail has been described elsewhere (Mir et al., 2014b).

Superoxide anion scavenging activity (alkaline pyrogallol iodometric assay): Alkaline pyrogallol mediated superoxide anion generation and antioxidant assays have been discussed elsewhere (Mir, 2015b). Test samples contained 0, 100 mg extract homogenate with 1 µmole of pyrogallol in 2 mL water while standards contained 0, 0.2 through 1 µmole of pyrogallol in 2 mL water.. Then each sample was added 1 mL 5% KI solution. The reaction was initiated with 0.1 mL $NaHCO_3$ solution followed by addition of 0.4 mL water, each sample allowed standing for 5 minutes and was added 0.5 mL 10% HCl each. The samples were allowed standing for about an hour and iodine content released in the samples was monitored at 450 nm. Blank samples containing pyrogallol as reagent blank were added 0.5 mL 10% HCl before adding bicarbonate solution to prevent generation of superoxide.

For organic phase extracts a typical assay consisted of 1.5 mL methanol containing 0, 0.2 through 0.6 µmole pyrogallol with 0.5 mL chloroform for standard samples while test samples contained 0.5 mL chloroform extract with or without 1 µmole pyrogallol in 1.5 mL methanol. Each sample was added 0.1 mL bicarbonate solution, allowed standing for 5 minutes. Additional increment(s) of 0.1 mL bicarbonate were added in case color contrast did not appear within few minutes of adding sodium bicarbonate solution. Each sample was added 1 mL methanolic KI solution, 0.5 mL dilute HCl and 2 mL water in that order, allowed standing 20-30 minutes, centrifuged at 3000 rpm for 5 minutes to separate out phases, and read supernatant at 450 nm for iodine release.

Superoxide anion scavenging activity (alkaline pyrogallol mediated ferrocyanide oxidation method): The test was applied to only water based extracts without pH adjustment. The extract equivalent of 75 mg homogenate was taken with 0 and 0.5 µmole of pyrogallol in 2.0 mL water. Standard samples contained pyrogallol 0, and 0.1 through 0.75 µmole in identical volume of water. Each sample was added 0.6 mL 0.01 M NaOH, allowed standing at room temperature for 20 minutes, added 0.4 mL potassium ferrocyanide

solution; allowed standing 5 minute and then added 0.5 mL 10% HCl. Absorbance was monitored after 20 minutes at 450 nm. The technique constitutes an additional method to monitor antioxidants for their ability to scavenge superoxide anion generated from alkaline pyrogallol (Mir, 2015b).

Superoxide anion scavenging activity (alkaline hydroxylamine mediated copper amine reduction method): Two mL water contained 0, 5 through 20 µmole hydroxylamine hydrochloride in standard samples while test samples contained up to 1 mL extract with 0 and 20 µmole hydroxylamine in 1 mL water. Each sample was added 1 mL 0.5 M ammonia solution; allowed standing 10 minutes followed by addition of 2 mL copper acetate solution (0.5 % w/v in water) to each with controls receiving 2 mL water instead. Stand for 10 to 15 minutes and read at 570 nm. The standardized method and its scope has been described elsewhere (Mir et al., 2013b).

Nitric oxide/nitrous acid scavenging activity (SNP mediated Griess reaction): *Haakh* homogenate extract 50 mg (APE) was incubated with 0 and 3 µmole SNP (as NO donor) with simultaneous standard containing 0, 1 through 10 µmole SNP in 2 mL water. Each sample was added 0.1 mL 1% sulfanilamide solution and 0.5 mL HCl, allowed standing in dark at room temperature for about an hour, added each 0.2 mL NEDA (0.2%) and 1.2 mL water. The samples were monitored at 540 nm after 30 to 40 minutes. Standard nitrite was run simultaneously under identical conditions using 0.01 through 0.1 µmole nitrite per sample. Scavenging activity of APE was determined in terms of standards of SNP and nitrite, and mass of nitrite generated over test range of SNP was also estimated with respect to nitrite calibration data. The assay in its various forms has been discussed in detail elsewhere (Mir, 2016c).

Nitric oxide scavenging activity (guaiacol oxidation method): The standard samples contained 2mL water with 0, 0.05 through 0.25 µmole nitrite, and test samples contained 0.5 mL test extract with 0.25 µmole nitrite in total 2 mL volume. Each sample was added 0.5 mL 10% HCl followed by 0.5 mL 1% guaiacol (w/v in 20% ethanol), and 1 mL water. The control extract samples containing nitrite contained matching volume of 20% ethanol instead of guaiacol. The samples were allowed standing at 25 ^{0}C in incubator for 25 to 30 minutes, and read at 450 nm. Nitrite reduced in extract treated samples was estimated in terms of standard calibration nitrite data. The technique constitutes an additional method to monitor antioxidants for their ability to scavenge nitrous acid/nitric oxide (Mir, 2016c).

RESULTS AND DISCUSSION

Considerations for taking the current research works have been three-fold: (i) to develop a battery of inexpensive *in vitro* antioxidant assays with applications within the reach of ordinary laboratories, (ii) to put these tests into applications with respect to indigenous plant materials and/or drugs which have been least studied for the antioxidant activities, and (iii) to provide a uniform unit of antioxidant potential in terms of µmole of active oxidant species reduced or scavenged per unit mass of test sample obviating use of arbitrary units such as per cent inhibition which is otherwise most commonly used in such studies.

As evident from the literature cited herein, a large number of simple techniques have been developed by the author, and tested for performance with respect to diverse collection of antioxidants. The standard unit of activity used therein has been uniformly put as envisaged. The present work constituted application of the published methods with few more additions towards the most common and popular vegetable of the valley, known locally as *Haakh*.

The extraction procedures were adopted to enable concentration of lipid-soluble antioxidants including principally carotenoids into chloroform-petroleum ether fraction (OPE), and water and alcohol soluble antioxidants principally phenolics including flavonoids and organic acidic constituents into hydroalcoholic fraction (APE). Alkaline pH for initial aqueous extraction was found to improve flavonoid solubility, and has been recommended for their extraction from plant materials (Windholz, 1983). Aqueous extraction of chloroform-ether fraction (OPE) was necessitated to enable extraction of organic thiocyanate forms, which are soluble in chloroform as well as in water (TE).

The pH of APE was found to be about 9.4. This was adjusted to nearly pH 6.7 using dilute HCl and dilute NaOH. During pH adjustment it was observed that the extract retained transparency over pH range 6.7 through 9.4, but turned opalescent at acidic pH 1.3 through 1.7.

Analyte content in Kashmiri *Haakh* homogenate extracts.

Thiocyanate: Thiocyanate content in chloroform soluble and water soluble extract from *Haakh* homogenate has been found to be 85 ± 2 µg g^{-1} using an improvised thiocyanate determination method (Basova et al., 2014). The method showed perfect linearity over test thiocyanate range of 20 through 100 µg in a 3 mL total

volume (r=0.999). Estimated thiocynate in *Haakh* is more than the levels reported for kohlrabi (30 µg g^{-1}) and lesser than those found in closely related cultivar kale (200 µg g^{-1}) (Gaitan, 1989). The content in kale has been reported to vary widely with about 150 µg g^{-1} fresh weight (Paxman & Hill, 1974) and about 95 through 1677 (µg g^{-1} dry matter) (Johnston & Jones, 1966; Chweya, 1988). Comparatively, lower values of isothiocyanate have been reported for fresh kale and fresh collard greens with mean concentrations respectively as 2.15 (range 0.2 through 7.5) and 3.4 (range 0.06 through 16.5) as µg g^{-1} fresh weight (Tang et al., 2013). In view of peculiar solubility features of TE, being soluble in chloroform and water, thiocyanate content in the present study may reflect sulforaphane-like compound in the TE of *Haakh* homogenate with estimated sulforaphane-like compound (mol wt 175.3) three times the mass of thiocyanate (mol wt 58) viz., 255 ± 6 µg g^{-1} (Table 1).

Phenolic acids: Gallic acid over the test range of 20 through 80 µg has shown perfect linearity (r=0.999). APE was estimated to contain phenolics as177±3 µg GAE g^{-1} homogenate (Table 1). Reported phenolic content in kale has ranged from about 285 µg g^{-1} dry matter (Ligor et al., 2013) to about 6 through 36 mg g^{-1} fresh matter (Ismail et al., 2004; Sikora & Bodziarczyk, 2012; Agarwal et al., 2017). Phenolic content in non-brassica wild *Haakh* genotypes (namely wuppal *Haakh* and pumb *Haakh*) in Kashmir has been reportedly in the range of 2.3 to 2.9 mg g^{-1} fresh weight (Murtaza et al., 2005).

Flavonoids: Employing HCl-method for determination of flavonoids (Mir et al., 2013, 2014) indicated rutin-like flavonoid(s) in AAE as 260 ±17 µg g^{-1} homogenate (Table 1). The assay technique has shown perfect linearity over test rutin range 20 through 100 µg (r=0.999). As evident from Table 1, the yield of flavonoids in APE was about 1.5 times more than the yield of phenolics. This is presumably because alkaline pH is considered most favorable to the extraction of most flavonoids (Windholz, 1983). Flavonoid content in kale by Aluminium method has been reported to vary from about 43 µg rutin-like g^{-1} dry matter as (Ligor et al., 2013) to about 14 mg quercetin-like g^{-1} (Agarwal et al., 2017).

Beta-carotene: The colorimetric assay has shown perfect linearity over test beta-carotene range 6 through 24 µg per tube in 3 mL chloroform at 465 nm. Estimated beta-carotene equivalent content that absorbs maximally at 465 nm in chloroform has been found to be 229 ± 9 µg g^{-1} homogenate (Table 1).The levels on an average are nearly 6.7 times more than those reported for raw collard greens with reported range from ca. 30 µg g^{-1} (Rudraappa, 2018) to ca. 38 µg g^{-1}(USDA, 2008), and nearly 3.6 times more than those reported for kale (Sikora & Bodziarczyk, 2012).

Table 1: Chemical constituents of Kashmiri *Haakh* extracts[a]

Parameter	Linear standard range[b]	µg g^{-1}	n	Extract
Flavonoid[c]	20 – 100 µg rutin	260 ± 17	5	APE
Phenolics [d]	20 – 80 µg gallic acid	177 ± 3	6	APE
Thiocyanate[e]	20 – 100 µg SCN (as KSCN)	85 ± 2	6	OPE[d]
Beta-carotene	6 – 24 µg beta-carotene	229 ± 9	5	OPE

The values are mean ± s.e. of n number of observations.
APE, alkaline phase (hydroalcoholic) extract; OPE, organic phase extract;
[a] Fresh leaf homogenate; [b] r >=0.99; [c] rutin-equivalent flavonoid;
[d]Gallic acid equivalent phenolics; [e]water soluble extract from organic phase extract residue presumably sulforaphane-like compound?

Antioxidant potential of *Kashmiri Haakh* homogenate extracts

Employing a battery of inexpensive in vitro antioxidant assays, an appreciable antioxidant potential has been demonstrated in the homogenate extracts. The activities in alkaline hydroalcoholic extract have ranged from general reduction of ions from permanganate, iodine, dichromate, ferric and of tetrazolium dye, MTT.

The relative potential order as determined by test assays with units µmole analyte reduced per g of *Haakh* homogenate extract (Table 1) (compared to standard ferric iron reducing potential considered valuable for determining total antioxidant potential of edible foods (Halvorsen et al., 2002) taken as unity for convenience of comparison) may be approximately put as follows :

PRP (55.3 X) >IRP (17.5 X) >DRP (5.6 X) >MTT (1.6 X) > FIRP (X)

FIRP in OPE is about 82 % of that in APE (P>0.1) implying that antioxidants are present in both hydroalcoholic and chloroform soluble extracts.

Antioxidant potential has been also demonstrated against special reactive oxygen species including active peroxide, hydroxyl radical, nitrous acid/nitric oxide and superoxide anion radicals from substrates like alkaline pyrogallol and alkaline hydroxylamine.

Peroxide scavenging potential of OPE was comparable to that of APE (Table 1, P>0.1) while peroxide scavenging activity of APE was about $1/12^{th}$ of the activity with respect to that of hydroxyl radical scavenging (P<0.01). Scavenging activity of APE against superoxide anion from alkaline pyrogallol yielded comparable values whether estimated by iodometric assay (1.64±0.12) or with ferrocyanide assay (1.50± 0.06)(P>0.1) while that in APE was nearly 12 times the activity found in OPE by iodometry (Table 1). APE extract when tested over 50 to 150 mg homogenate

Table 2: Antioxidant potential of Kashmiri Haakh extracts[a]

Parameter	Linear range target oxidant[b], µmole	Values	n	Extract
Permanganate reducing potential	0.1 – 1.0	37.6±1.9	6	APE
Iodine reducing potential	0.3 – 5.0	11.9 ± 0.8	6	APE
Dichromate reducing potential	0.5 – 3.0	3.8 ± 0.4	6	APE
Ferric iron reducing potential (thiocyanate method)	0.1 – 0.5	0.68 ± 0.08	5	APE
	0.6 – 3.0	0.56 ±0.05	6	OPE
MTT reducing potential[c]	0.241 (=100 µg)	1.08 ± 0.07	6	APE
Peroxide scavenging potential (iodometric)	0.5 – 2.5	0.40 ± 0.08	5	APE
		0.42 ± 0.02	6	OPE
Hydroxyl radical scavenging potential (Fenton-catechol)	0.25 – 2.5	4.99± 0.07	6	APE
Superoxide anion scavenging potential (AP-I)[d]	0.2 – 1.0	1.64± 0.12	6	APE
	0.2 – 0.6	0.14 ± 0.02	6	OPE
Superoxide anion scavenging potential (AP-F)[e]	0.1 – 0.75	1.50 ± 0.06	6	APE

Superoxide anion scavenging potential (AH-C)[f]	5 - 20	16.2 ± 3.5	5	APE
Nitrous acid scavenging potential (diazo method)	1-10 (SNP)	46.4±1.8[g]	5	APE
	0.01 – 1.0 (NO_2)	0.69 ± 0.03[h]	5	APE
Nitrous acid scavenging potential (guaiacol method)	0.05 – 0.25 (NO_2)	0.63 ± 0.04	5	APE

[a] µmole reduction g^{-1} fresh leaf homogenate basis; [b] perfect linearity over test range wherever applicable (r >=0.99); [c] estimate reflecting formazan produced or MTT reduced; [d]AP-I, alkaline pyrogallol-iodometric; [e]AP-F, alkaline pyrogallol-ferrocyanide method; [f]AH-C, alkaline hydroxylamine - copper method; [g] SNP equivalent (each µmole SNP generated estimated 0.015 µmole nitrous acid under test conditions); [h] Nitrous acid equivalent. APE, alkaline phase (hydroalcoholic) extract; OPE, organic phase extract; n, number of observations per parameter

equivalent failed to reduce copper when incubated with 50 µmole copper (II). This observation enabled monitoring ability of APE on superoxide anion scavenging activity with alkaline hydroxylamine coupled copper reduction assay demonstrating thereof about 10 times more values than mean of values obtained with alkaline pyrogallol ($P<0.1$). APE showed appreciable nitrous acid scavenging activity with values obtained with diazotization method similar to those obtained
with guaiacol method ($P>0.1$). As evident (Table 1), when scavenging activity was estimated in terms of SNP, the activity was nearly 67 times that of nitrite. This was due to the fact that under test conditions, SNP over the test range of 1 through 5 µmole showed perfect linearity in nitrous acid formation ($r=0.999$) with each µmole SNP generating 0.015 µmole of nitrite.

The preceding assay systems have demonstrated that the *Haakh* extracts are capable of not only strong reducing activity against common oxidant ions but have also an efficient scavenging activity against different reactive oxygen species, thus ensuring a valuable place for the vegetable in the local diet. On technical considerations, the work has added two more antioxidant assay systems including guaiacol method for monitoring nitrous acid/nitric oxide scavenging activity, and alkaline-pyrogallol mediated ferrocyanide oxidation for monitoring superoxide anion scavenging activity.

CONCLUSION

The present investigation has revealed appreciable antioxidant potential in the extracts of common Kashmiri *Haakh* using a battery of *in vitro* assay systems to evaluate activities that involve direct reduction of ionic species as well as those involving activity against special reactive oxygen species. The activities are apparently related to its known phytoconstituents. The work has shown appreciable concentration of flavonoids, phenolics, thiocyanate and beta-carotene in the *Haakh* extracts. Besides, the work has introduced two newer assay systems, respectively, for monitoring nitrous acid/nitric oxide, and superoxide anion from alkaline pyrogallol.

REFERENCES

1. Nierenberg D. 2012; https://weblogtheworld.com/formats/featured/history-of-collard-greens-extends-far-beyond-north-america
2. Wikipedia. 2017; https://en.m.wikipedia.org/wiki/collard-greens
3. Mateljan G. 2018; George Matelijan Foundation; https://www.whfoods.com/genpage.php?tname=foodspice&dbid=138

4. Kak AM. Ethnobotany of Kashmiris, March 1983, Part III, page 9; Reprinted from Kahkashan XVI (1):1-21.
5. Ware M. Medical News Today; 2016. https://www.medicalnewstoday.com/articles/277957.php
6. Adams J.2018; https://www.naturalfoodseries.com/11-health-benefits-collard-greens/
7. Packer L, Witt EH, Tritschler HJ. Radic Biol Med.1995; 19(2):227-250.
8. USDA. National Nutrient database for Standard Reference, Release 21, Collard greens raw; 2008; https://www.nal.usda.gov/fnic/foodcomp/cgi-bin/list_nut_edit.pl
9. Tang L, Paonessa JD, Zhang Y, Ambrosone CB, McCann SE. J Funct Foods. 2013; 5(4): 1996–2001.
10. Lixandru M. 2015; https://www.natureword.com/properties-and-benefits-of-collard-greens/
11. Rudraappa U. 2018; https://www.nutrition-and-you.com/collard-greens.html
12. Melo EA, Maciel MIS, Lima VLAG, Leal FLL, Caetano ACS, Nascimento RJ. Ciênc. Tecnol.Aliment. [Online]. 2006; 26(3): 639-644.
13. Clifford A, Dawson P. J Fd Res 2012; 1(4):66-73.
14. Arnao MB,Sanchez-Bravo J, Acosta M. IUBMB Life,2008; 39(6):1125-1134.
15. Ligor M,Trziska T, Buszewski B. Food Anal. Meth. 2013; 6: 630-636.
16. Agarwal A, Raj N, Chaturvedi N. Int. Jr. Adv. Res. Biol. Sci., 2017; 4(4):22-29.
17. Ismail A, Marjan ZM,Foong CW. Food Chem. 2004; 87:581-586.
18. Silkora E, Bodziarczyk I. Acta Sci. Pol. Technol. Aliment, 2012; 11(3):239-248.
19. Halvorsen BL,Holte K, Myhrstad MCW, Barikmo I, Hvattum E,Remberg F, Wold A-B, Haffner K, Bauggerod H,Andersen LF, Moskaug O,. Jacobs DR, Jr., Blomhoff R. J. Nutrition. 2002;132:461-471.
20. Murtaza I, Beigh GM, Shah TA, Hussain A, Khan AA, Kaur C. Jr Plant Biochem. Biotech 2005; 14(2): 215-218.
21. Pourmorad F, Hosseinimehr SJ, Shahabimajd N. African Journal Biotechnology 2006; 5(11):1142-1145.
22. John B, Sulaiman CT, George S, Reddy VRK. International Journal of Pharmacy and Pharmaceutical Sciences, 2014; 6(1): 406-408.
23. Mir SA, Ahangar AA, Bhat AS. International Journal Pharm Tech Research, 2013a; 5(2): 341-348.
24. Mir SA, Bhat AS, Ahangar AA. International Journal Pharm Tech Research, 2014a; 6(2), 751-758.
25. Basova EM,Ivanov VM, Apendeeva OK. Moscow University Chemistry Bulletin. 2014; 69(1):12-19.
26. Simpson KL, Tsou SCS, Chichester CO: In: Augustin J, Klein BP, Becker DA, Venugopal PB (Eds.).Methods of vitamin assay, 4th ed., John Wiley and Sons, N.Y, 1985; pp.185-220.
27. Mir SA. International Journal Pharm Tech Research, 2016a; 9(1): 50-59.
28. Mir SA. International Journal Pharm Tech Research, 2016b; 9(1): 70-78.
29. Mir SA. International Journal Pharm Tech Research, 2015a; 7(2): 276-286.
30. Mir SA, Bhat AS, Ahangar AA. International Journal Pharm Tech Research, 2014b; 6(2):734-742.
31. Madesh M, Balasubramanian K.A. Indian Journal Biochemistry & Biophysics. 1998; 35:184-188.
32. Mir SA, Bhat AS, Ahangar AA. International Journal Pharm Tech Research, 2014b; 6(2): 759-768.
33. Mir SA. International Journal Pharm Tech Research, 2015b; 7(2): 266-274.
34. Mir SA, Bhat AS, Ahangar AA. International Journal Pharm Tech Research, 2013b; 5(2):349-358.
35. Mir SA. International Journal Pharm Tech Research, 2016c; 9(1): 60-69.
36. Windholz M (Ed.). The Merck Index. 10th Edn, Merck & Co., Inc, N.J., USA, 1983.
37. Gaitan E (Ed.).Environmental goitrogenesis. CRC Press, USA, pp. 18-19, 1989.

38. Paxman PJ, Hill R. J Sci Food Agric. 1974; 25(3): 323-328.
39. Johnston TD, Jones IH. J Sci Food Agric. 1966; 17(2):70-71.
40. Chweya JA. Acta Hortic. 1988; 218:181-190..https://www.ishs.org/ishs-article/218_23