Analytical and Biological Studies of *Kanji* and Extracts of its Ingredient, *Daucus carota* L.

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Summary: A fermented beverage, *Kanji,* prepared from roots of *Daucus carota* L. subsp. s*ativus* (Hoffm.) Arcang. var. *vavilovii* Mazk. (*Apiaceae*), despite long usage history has not been investigated for analytical studies and biological activities. Therefore, the present study aimed to investigate different types of *Kanji* samples and various types of extracts/fractions of root of the plant for a number of analytical studies and *in vitro* antioxidant activities. The *Kanji* sample, Lab-made *Kanji*, having better analytical and biological profile was further investigated for preliminary clinical studies. The analytical studies indicated that Lab-made *Kanji* was having comparatively higher contents of phytochemicals than that of the commercial *Kanji* samples, different types of extracts and fractions ($P < 0.05$). All the *Kanji* samples and aqueous and ethanol extracts of fresh roots exhibited comparable antioxidant activities in DPPH assay (52.20 - 54.19%) that were higher than that of methanol extract (48.78%) of dried roots. The antiradical powers ($1/ EC_{50}$) of Lab-made *Kanji* and aqueous extract were found to be higher than that of the ethanol and methanol extracts. In β-carotene linoleate assay, the *Kanji* samples showed higher activity than that of the methanol extract, but comparable to that of the vitamin-E and butylated hydroxyl anisole (BHA) ($P < 0.05$). A preliminary clinical evaluation indicated that *Kanji* has no harmful effect on blood components, liver function and serum lipid profile. The results of the present study indicate that *Kanji* is an effective antioxidant beverage.

Keywords: *Kanji*; *Daucus carota* L.; *Apiaceae*; Antioxidant activity; phytochemical constituents, Clinical evaluation

Introduction

Beside nutritional significance, vegetables due to their antioxidant properties can reduce the incidence of mortality rate of ailments involving oxidative stress such as cancer and cardio- and cerebro-vascular diseases [1]. Due to nutritional status, vegetables have varying popularity among people of different age groups throughout the world. Carrots are among those vegetables which are found and consumed in almost all parts of the world. On the basis of nutrition, these have been ranked as 10th among 39 fruits and vegetables as per the United States Agricultural Statistics [2]. The continuous research and proven health benefits of carrots are increasing their consumption and popularity day by day [3]. This vegetable has a number of cultivars – different colored carrots, subspecies and varieties, which are distributed in different parts of the world. Based on distribution, carrots are mainly grouped as Western carrots (yellow) and Eastern carrots (Black).

Daucus carota L. subsp. s*ativus* (Hoffm.) Arcang (Black carrot) has six varieties; yellow (var. *scharrovii* Mazk.), violet (var. *biossieerii* Schweinf.), pink (var. *rosseus* Mazk.), orange (var. *zhukovskii* Setch.), white (var. *albus* Alef.) and black (var. *vavilovii* Mazk.). The roots of the variety *vavilovii* Mazk are used to prepare a fermented beverage in some Asian countries particularly, Pakistan and India, whereby it is known as *Kanji.* It is a quite popular remedy for the treatment of indigestion, loss of appetite and liver disorders. It is prepared and used extensively as an appetizer in early summer season [4]. Since long, it is being prepared both in homes as a house-hold remedy and on a small industrial scale by road-side vendors. Despite such long usage history, it is neither characterized analytically nor evaluated biologically, which necessitates the investigation of this remedy to make it an evidence-based product.

The tuberous roots of black carrots, the main ingredient of *Kanji*, possess diuretic, digestive tract soothing, hepatoprotective and uterine stimulating properties [5, 6]. These are reported to have menstruation delaying and uterine contraction inducing effects [7]. The decoction of roots is efficacious in removing urinary tract stones [8]. The use of infusion of roots for treating edema, flatulence, indigestion and menstrual problems are other traditional claims [5].

The literature review indicated a number of pharmacological studies on roots of different cultivars of the plant. Two studies described the

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antioxidant activity of carrots using *in vitro* models [9, 10]. The seven different colored verities of carrots were biofortified to enhance their antioxidant potential, which was then evaluated using DPPH and (2, 2- azino-di [3-ethylbenzthiazoline-6- sulfonate] ABTS models [11]. Moreover, the juice of fresh roots had shown hepatoprotective activity in CCl_4 and lindane–induced intoxicated mice and rats [12, 13]. Surprisingly, all such reports are lacking in the correct identification of the subspecies or the variety used. Likewise, existing literature lacks reports on the chemical characterization and biological activity studies of *Kanji*.

Therefore, the present study aimed to investigate various types of *Kanji* samples*,* extracts and fractions of roots of *Daucus carota* L. subsp. s*ativus* (Hoffm.) Arcang. var. *vavilovii* Mazk. for chemical characterization and antioxidant activity studies. It was further aimed to investigate well-characterized Lab-made *Kanji* in healthy individuals to find its effect on blood components, liver function and serum lipid profile. The findings of this study may provide analytical profiles prerequisite for standardization and scientific evidence to folklore claims of *Kanji.*

Results and Discussion

The results for the estimation of total contents of primary and secondary metabolites of different *Kanji* samples, extracts and fractions are given in Table-1. The linear regression equations used for the determining different groups of metabolites were as total protein ($y = 1.2176x - 0.0031$, $R^2 = 0.9984$), total polyphenols ($y = 0.1520x - 0.0009$, $R^2 = 0.9996$), total polysaccharides ($y = 9.4258x - 0.011$, $R^2 = 0.9874$) and total flavonoids ($y = 15.981x - 0.0182$, $R^2 = 0.991$). These results indicate that the phytochemicals in Lab-made *Kanji* are significantly higher than that of its other types ($P < 0.05$). However, phytochemical constituents of all the commercial *Kanji* samples are alike. Total protein contents of Lab-made *Kanji* are significantly higher than other *Kanji* samples, extracts and fractions, except methanol extract ($P < 0.05$). Total polysaccharides, polyphenols, flavonoids and glycosaponins of all the *Kanji* samples are significantly higher than that of all the extracts and fractions ($P < 0.05$). Contrary to previous report, total phenol contents of ethanol extract of fresh root were found to be higher [14], which might be due to different cultivar, variety, growing conditions and age of the plant used in the present study.

The results of total solids (g/mL) and pH of Lab-made, homemade and six commercial samples of *Kanji*, without having the additional table salt or black salt, are presented in Fig. 1. All the *Kanji* samples were found to be having no significant difference in pH. On the other hand, the total solid contents of Lab-made, homemade and commercial samples (C5 and C6) of *Kanji* were similar, however higher than that of the other types of commercial *Kanji* samples ($P < 0.05$). Interestingly, all the samples of *Kanji* showed variation in weight/mL, which could be attributed to a number of probabilities such as non-availability of standard preparation protocol, addition of extra water to increase volume to maximize profit, ice cubes to keep it cool for the preservation of palatability, and use of different carrot variety. Moreover, the samples showing less total solid contents were light as well as hazy in color, whereas Lab-made and homemade *Kanji* samples were crystal clear having bright purple color. To rule-out the possibility of aforesaid factors, uv/visible profiles (scans from 800-200 nm) of all the *Kanji* samples were compared to each other. The overlay scan of different *Kanji* samples*,* extracts and fractions are given in Fig. 2. The similarity in metabolomics profiles of all the *Kanji* samples confirmed that the same carrots variety was used in the preparation of this beverage. However, the difference of peak intensities in metabolomics fingerprints was the clear evidence of using different quantities of carrots in the preparation of this drink or dilution made at the time of selling.

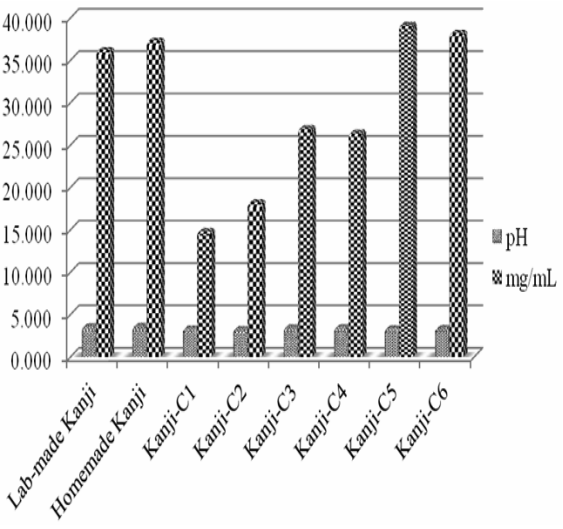


Fig. 1: Total solid contents (g/mL) and pH of different types of *Kanji* samples. C1- C6 (commercial samples).

Table-1: Total contents (mg/g) of phytochemicals of different types of *Kanji* and various types of extracts of root of *Daucus carota* L., and fractions of sequential methanol extract.

Sample	Total proteins	Total polysaccharides	Total polyphenols	Total flavonoids	Total glycosaponins
***Kanji* (Lab-made)**	**20.61**	**6.66**	**8.58**	**7.21**	**201.5**
***Kanji* (Home-made)**	**18.77**	**5.96**	**7.27**	**6.73**	**188.98**
***Kanji* (Com-1)**	**18.67**	**5.78**	**7.87**	**6.87**	**184.18**
***Kanji* (Com-2)**	**18.33**	**5.26**	**7.75**	**6.79**	**186.85**
***Kanji* (Com-3)**	**17.22**	**5.46**	**7.48**	**6.27**	**187.44**
***Kanji* (Com-4)**	**18.79**	**5.68**	**6.97**	**6.48**	**185.55**
***Kanji* (Com-5)**	**17.22**	**5.13**	**6.47**	**5.97**	**187.18**
***Kanji* (Com-6)**	**18.27**	**5.86**	**7.28**	**6.58**	**185.82**
Aqueous extract	**23.34**	**10.30**	**9.51**	**9.97**	**235.5**
Ethanol extract	**23.89**	**11.81**	**9.78**	**10.29**	**201.2**
Hexane extract	**0.69**	**1.43**	**2.19**	**0.64**	**0.00**
Chloroform extract	**1.97**	**3.29**	**2.86**	**1.70**	**0.40**
Methanol extract	**20.71**	**0.2**	**9.31**	**8.17**	**19.20**
Hexane fraction	**1.05**	**0.82**	**0.39**	**5.94**	**0.00**
Chloroform fraction	**0.94**	**0.88**	**1.32**	**0.95**	**0.35**
Ethyl acetate fraction	**4.04**	**0.97**	**4.39**	**2.02**	**0.42**
n-butanol fraction	**7.07**	**5.85**	**4.92**	**3.39**	**108**
Water fraction	**11.39**	**9.37**	**5.05**	**6.37**	**112**

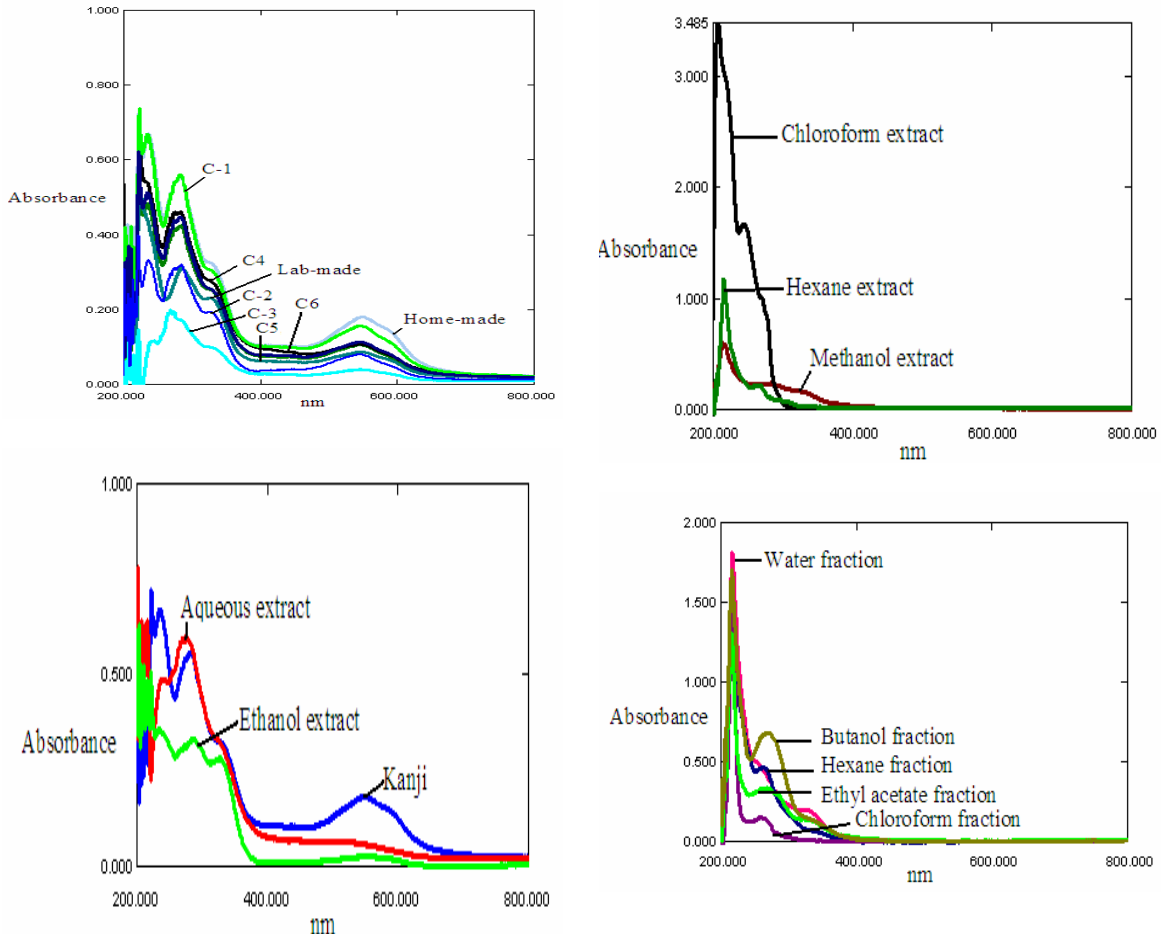


Fig. 2: UV/Visible scans of different types of *Kanji* samples, extracts and fraction of root of *Daucus carota* L. in a range of 800-200 nm. A [scans of Kanji samples, C1-C6 (commercial samples)]; B (*Kanji* and aqueous and ethanol extracts); C (sequential extracts); D (fractions of methanol extract).

The HPTLC profiles of all the *Kanji* samples and aqueous extract of fresh roots did not show any band under UV exposure, both at short wavelength (254 nm) and long wavelength (366 nm). Contrary to this ethanol extract of fresh roots and different types of extracts and fractions of dried roots showed bands at both the wavelengths. The absence of bands in *Kanji* and aqueous extract may be due to lower concentration of compounds, active at both the wavelengths.

The results of *in vitro* antioxidant activity of different *Kanji* samples, extracts and fractions of sequentially obtained methanol extract, determined by DPPH assay, are shown in Fig. 3. The antioxidant activities of all *Kanji* samples, ethanol extract and aqueous extract were not statistically different ($P > 0.05$). Likewise, all the extracts of dried material obtained by sequential extraction were not having any significant difference of antioxidant activity. The antioxidant profile of hexane, chloroform, ethyl acetate, n-butanol and water fractions of methanol extract was also found to be similar. However, activity of all the *Kanji* samples, extracts and fractions was lesser than that of the standards *viz* BHA, QTN and vitamin E ($P < 0.05$).

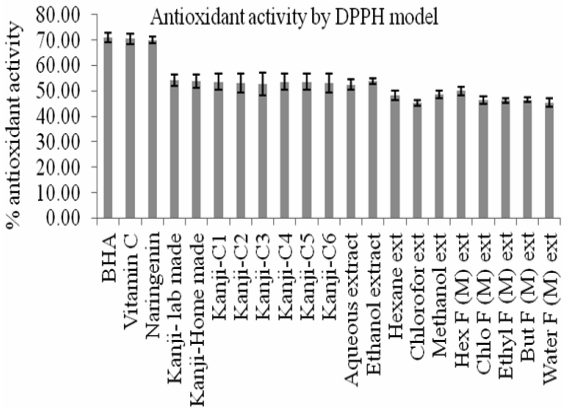
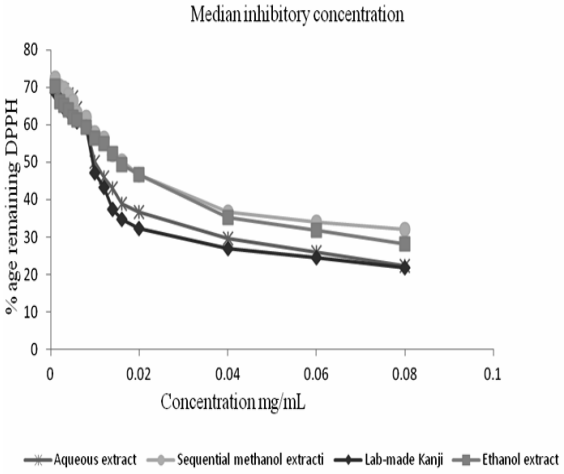


Fig. 3: Antioxidant activity of standards and different types of *Kanji* samples, and extracts and fraction of root of *Daucus carota* L. using DPPH assay (n=3). BHA (butylated hydroxy anisole); C1-C6 (commercial samples); Ext (extract); Hex F (hexane fraction); Chlo F (chloroform fraction); Ethyl F (ethyl acetate fraction); But F (butanol fraction); M (methanol)

Lab-made *Kanji*, aqueous and ethanol extracts of fresh root and sequential methanol extract of the dried root material were subjected to dose dependent studies using DPPH assay to determone their EC_{50s} and antiradical powers ($1/EC_{50}$). The plot of various concentrations of the samples and the percentage remaining DPPH is shown in Fig. 4, which indicates that Lab-made *Kanji* and aqueous extract have almost equal EC_{50} and antiradical power (0.01 mg/mL, 100), whereas ethanol extract and sequential methanol extract have silimlar EC_{50} and antiradical power (0.016 mg/mL, 62.50). Hence, lab-made *Kanji* and aqueous extract exhibited powerful antioxidants activity than that of the ethanol and methanol extracts.



Fig, 4: Median inhibitory concentrations (EC_{50s}) of Lab-made *Kanji*, sequentially obtained methanol extract, ethanol and aqueous extract of root of *Daucus carota* L.

The results of antioxidant activities of *Kanji* samples and different types of extracts as well as various fractions of sequential methanol extract, determined by β-carotene assay, are shown in Fig. 5. These results indicated that all the *Kanji* samples had comparable antioxidant activity. The ethanol and aqueous extracts of fresh roots were also comparable to each other. In sequential extracts, hexane and chloroform extracts exhibited higher activity than that of the methanol extracts, which was due to extraction of carotenoids in nonpolar as well as slightly polar solvents. The activity of chloroform, ethyl acetate and water fractions was also comparable to each other. The degradation kinetics of *Kanji* samples, extracts and fractions are given in Fig. 6 and 7. These results clearly show that the absorbance of the control- free from antioxidant- is deceasing rapidly due to bleaching of β-carotene as compared to the emulsion containing *Kanji*, extracts, fractions and the standards. The protection to peroxidation provided by the Lab-made *Kanji* was comparable to that of vitamin-E and BHA. *Kanji* samples as well as

extracts and fractions have inhibited the peroxidation of linoleic acid due to the presence components having oxygen radical absorbing capacity, as reported previously for carrots [10]. It was also reported that antioxidant activity- using β-carotene assay of carrot juice kept at 2°C was increased until 30 min [9].

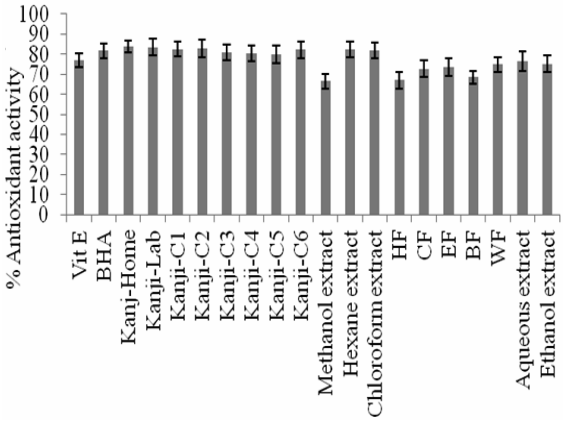


Fig. 5: Antioxidant activity of standards and different types of *Kanji samples,* extracts and fraction of root of *Daucus carota* L. using β-carotene assay (n=3). Vit E (vitamin E); BHA (butylated hydroxy anisole); C1-C6 (commercial samples); HF (hexane fraction); CF (chloroform fraction); EF (ethyl acetate fraction); BF (butanol fraction); WF (water fraction).

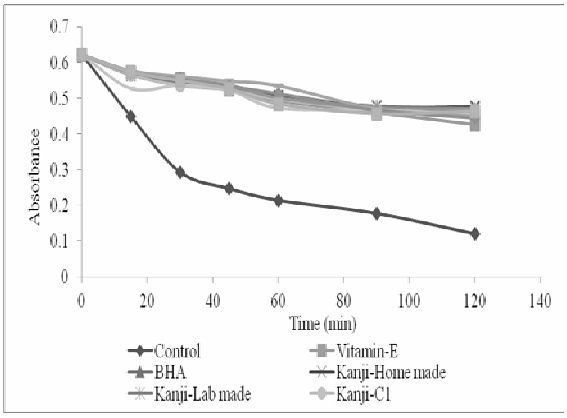


Fig. 6: Degradation kinetics of β-carotene in control and samples containing standards and different types of *Kanji* samples using β-carotene linoleate model (n=3). BHA (butylated hydroxy anisole); C (commercial).

The Pearson correlation analysis indicated that antioxidant activities of all the samples were correlated to their polysaccharide contents ($P < 0.05$) and total polyphenol and flavonoid contents ($P < 0.01$). Interestingly, the phytochemical constituents responsible for antioxidant activity were found to be present in all types of extracts, used in this experiment. The comparable profiles of aqueous and ethanol extracts indicate that water is a good solvent for the preparation of *Kanji*. The distribution of antioxidant components in hydrophilic and hydrophobic solvent extracts is supported by a previous study, wherein antioxidant activities determined using DPPH and ABTS assays are found in both types of solvent extracts [11]. The slightly higher activity of the ethanol extract was also in accordance to the reported previously [14].

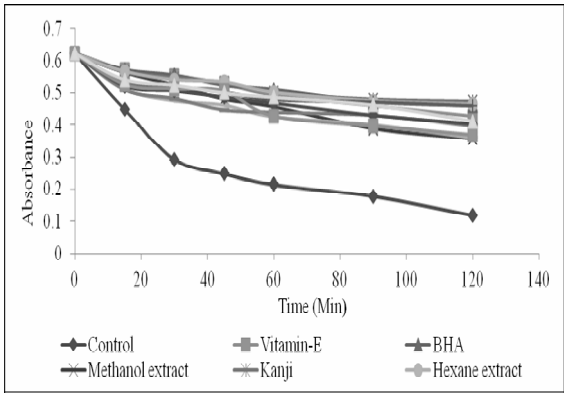


Fig. 7: Degradation kinetics of β-carotene in control and samples containing standards, extracts and fractions of fraction of root of *Daucus carota* L. using β-carotene linoleate model (n=3). BHA (butylated hydroxy anisole); HF (hexane fraction); CF (chloroform fraction); EF (ethyl acetate fraction); BF (butanol fraction); WF (water fraction).

The results of preliminary clinical study of chemically and biologically characterized Lab-made *Kanji* are shown in Fig. 8. These results show that except neutrophil count there is not any significant difference in blood components, liver function markers and serum lipid profile before and after the treatment. The presence of all the determined clinical parametrs within the normal limits indicate the usefulness of *Kanji*. However, all the individauls under study revealed certain additional benefits such as increase in appetite and urination after drinking *Kanji*. The increased urine volume was further found to be relieving burning sensation of urethera during mictuartion, very common problem in summer season. There was no change in body weight of all the volunteers during two weeks of the study. This data lend evidence that the use of *Kanji* is beneficail

and without harm. This is the first report of preliminary clinical evaluation of *Kanji*. However, as stated earlier, the extracts of black carrots had hepatoprotective effects in rodents [12, 13]. The findings of the present study may provide evidence to conduct a full scale clinical investigations on *Kanji* in patients suffering from liver disorders.

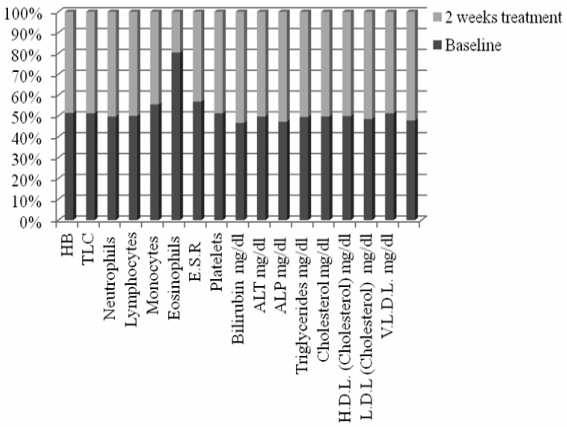


Fig. 8: Comparison of hematology, liver function test and lipid profile of male adult healthy volunteers (n=6) consuming 240 mL *Kanji*/day for two weeks.

Experimental

Chemicals and Solvents

The chemicals procured from Sigma Aldrich included 1, 1-diphenyl-2-picryl hydrazyl (DPPH), β-carotene, linoleic acid, butylated hydroxyl anisole (BHA), tocopherol (vitamin E), naringenin, gallic acid, bovine serum albumin, quercetin (QTN) and, folin ciocalteau's reagent. The solvents/chemicals of E-Merck, purchased from the local market included ethanol, methanol, chloroform, hexane, ethyl acetate, n-butanol, sodium carbonate, copper sulphate, sodium hydroxide, potassium sodium tartrate, aluminum nitrate, enthrone reagent and glucose.

Plant Material, Extraction and Fractionation

The black carrots, purchased in the month of March from a local vegetable market were authenticated by Professor Dr. Zaheer ud Din Khan, Department of Botany, Government College University, Lahore, Pakistan. A voucher specimen was deposited in herbarium of Department of Botany, Government College University, Lahore, Pakistan, vide reference No. G. C. Bot. Herb. 958.

The roots were washed with water to remove extraneous matter and the residual water was dried in air. The material was divided into three portions; one to be used after drying whereas two to be used as fresh.

The fresh sliced material (250 g) was macerated overnight separately in 500 mL of ethanol and water. The extracts were collected and the residues were extracted again using the same procedure. Ethanol extracts were pooled and solvent was removed *in vacuo* at 40^{o}C, whereas the pooled aqueous extracts were dried using freeze dryer (Christ, Germany).

The second portion of the material was crushed and dried under shade. The dried material (800 g) was extracted sequentially using solvents such as petroleum ether, chloroform and methanol in soxhlet apparatus. All the extracts were dried *in vacuo* at 40^{o}C. Based on the yield, methanol extract was fractionated by partitioning using solvents such as hexane, chloroform, ethyl acetate, butanol and water. Except water fraction (dried in freeze dryer), all the fractions were dried *in vacuo* at 40 ^{o}C.

Preparation of Kanji and Collection of Samples from Market

The 3rd portion of the fresh roots was used to prepare *Kanji* which was termed as Lab-made *Kanji*. It was prepared using the most commonly used traditional recipe as 113 g vertically sliced thin-long pieces of roots and 5 g each of red chilies, mustard seeds and table salt (sodium chloride) were added in a glass jar containing 1500 ml water. The container was covered and the contents were allowed to ferment spontaneously at room temperature for 4 days [15, 16]. Afterwards, it was stored in refrigerator (10 - 15^{o}C) to be used whenever needed. The other ingredients, red chilies, mustard seeds and salt are added to impart good taste.

Six *Kanji* samples, each 250 mL, were randomly purchased from roadside vendors asking them not to add any other ingredient. These samples were designated as C1, C2, C3, C4, C5 and C6. Another 250 mL *Kanji* sample was collected from home of a resident of Lahore, Pakistan, which was labeled as homemade *Kanji*.

Analytical Studies

Estimation of Total Proteins (TP): Total protein was estimated by method of Lowry *et al.* [17], briefly 50 mg each of the samples was dissolved in 10 mL distilled water in a centrifuge tube using vortex. The tubes were centrifuged for 10 min at

2700 rpm and 0.1 mL aliquots of supernatant were transferred in test tubes and made the volume 1 mL with distilled water. The contents were mixed after adding 3 mL of reagent C – made by mixing 50 mL of reagent A (2% sodium carbonate in 0.1N sodium hydroxide) and 1 mL of reagent B (0.5% copper sulfate in 1% potassium sodium tartrate) – and 0.2 mL of folin-ciocalteau reagent. The tubes were incubated at room temperature for 30 min and absorbance was measured at 600 nm (UV-2550, Shimadzu Corporation Japan) against a blank having all the reagents except the sample(s). Bovine serum albumin (Fraction V) solutions in a concentration range 12.50-100 µg/mL, treated like the samples, were used to plot a standard curve for the determination of TP.

Estimation of Total Polysaccharides: Each of the samples (0.2 g) was dissolved in 7 mL of 80% hot ethanol in a centrifuge tube to remove soluble sugars. After vortex for 2 min, the tubes were centrifuged at 2700 rpm for 10 min and supernatant was collected. The procedure was repeated with the residue until washing did not give color with enthrone reagent. Then the final residue was dried on water bath, mixed with 10 mL mixture of distilled water and 25% HCl (1: 1 v/v) at 0 ^{o}C for 20 min, and then centrifuged at 2700 rpm for 10 min to collect supernatant. The extraction was performed twice and the supernatants were pooled, and the volume was made 100 mL with distilled water. Aliquots (0.1 mL) were taken in test tubes and made the volume 1 mL with distilled water, then 4 mL enthrone reagent was added. The contents were mixed, heated in boiling water bath for 8 min, cooled rapidly and the intensity of green color produced was measured at 630 nm against a blank having all the reagents except the sample. Glucose solutions having concentration 20, 40, 60, 100 and 200 µg/mL, treated like samples were used to plot the standard curve. The glucose concentration calculated from the standard curve was multiplied by factor 0.9 to determine total polysaccharides [18, 19].

Estimation of Total Glycosaponins: Total glycosaponins were determined as described by Hussain *et al*. [20], briefly, 1 g of each sample was refluxed in 50 mL methanol for 30 min and extract was collected. The process was repeated again and the pooled extract was concentrated to 10 mL using rotary evaporator, which was then added drop wise in a tarred beaker containing 50 mL acetone. The precipitate was dried in an oven at 100 ^{o}C, cooled to room temperature and weighed to determine total glycosaponins using the following equation.

Glycosaponins = (Weight of precipitate / Weight of sample) X 100

Estimation of Total Polyphenols: Total polyphenol contents were estimated by a method of Singleton and Slinkard [21] with some modifications. Gallic acid solutions of different concentrations were prepared in methanol to be used as standards. Each of the samples/standard solutions (0.2 mL) was taken in a test tube and made the volume 1 mL with methanol. Then after adding 2 mL of folin-ciocalteu reagent the contents were mixed thoroughly. After 4 min, 2 mL of 15% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h at room temperature. The absorbance of each of the mixtures was measured at 760 nm against a blank prepared in the same way as that of the samples, except the addition of sample or standard. Total polyphenol contents (mg of gallic acid equivalents) in samples were determined from calibration curve of gallic acid using linear regression.

Estimation of Total Flavonoids: Total flavonoids were estimated by method of Chang *et al.* [22] with some modifications. Briefly, quercetin (QTN) solutions of different concentrations were prepared in methanol to be used as standards. Each of the samples/standard solutions (0.2 mL) was taken in test tube and made the volume 1 mL with methanol, then 0.1 mL of 10% aluminum nitrate solution, 0.1 mL of 1M potassium acetate and 4.6 mL distilled water were added. The contents were mixed thoroughly and allowed to incubate for 45 min at room temperature. The absorbance was measured at 415 nm against a blank, prepared in the same way, except the addition of sample/standard. The flavonoid contents (mg of quercetin equivalents) were determined from the calibration curve of QTN using linear regression.

Determination of Total Solid (Weight/mL) and pH of Kanji: All the *Kanji* samples were used to calculate their total solid contents using pycnometer of 25 mL capacity. The same samples were used to determine their pH using pH meter (WTW, Inolab, Germany).

UV/Visible Profiling of Kanji, Extracts and Fractions: Fifty milliliters of each of *Kanji* samples was taken in a test tube and volume was made 3mL with methanol and the resulting sample was scanned in a range of 800 – 200 nm using methanol as a blank. The spectra obtained were compared to each other to find similarity or difference in their chemical composition.

Five hundred microliters of solution of each of the extracts and fractions having concentration 1 mg/mL were taken in test tube and volume was made 3 mL with methanol. Then each of the samples was scanned as mentioned above to get overlay of spectra.

HPTLC of Kanji, Extracts and Fractions: The samples of *Kanji* and aqueous extracts (0.1 mg/mL) were analyzed using HPTLC system of CAMAG, Berlin, Germany, equipped with TLC plate scanner (Model-3), semi- automatic sampler (Linomat-5), 254/366 nm image recorder (PROSTER 3) and winCATS 4 software. The densitograms obtained were compared to each other.

In Vitro Biological Studies

Free Radical Scavenging Activity by DPPH Model: Each of the freeze-dried *Kanji* sample, extracts, fractions and the standards such as BHA, QTN and vitamin E was dissolved in methanol to get final solution of concentration 0.1 mg/mL.

The DPPH assay was performed as described by Brand-Williams *et al.* [23] and *Robards et al.* [24], briefly 2 mL of 0.1 mM methanolic solution of DPPH was mixed with 200 µL of each of the test samples/standards in a test tube. Then the volume was made 3 mL with methanol, the contents were mixed and all the tube was kept at room temperature for 60 min. A mixture of 2 mL the DPPH solution and 1 mL methanol, treated like sample/standard served as a control. The absorbance of all the samples, standards and the control was measured at 515 nm against methanol as a blank. Free radical scavenging activity (FRSA) was determined using the following equation.

$$FRSA = [(Ac - As) / Ac] X 100$$

Where "Ac" is absorbance of the control and "As" is the absorbance of the tested sample after 60 min.

Antiradical Power by DPPH Assay: The Lab-made *Kanji,* aqueous and ethanol extracts of fresh roots and methanol extract of dried material were further investigated for dose-response relationship and determination of anti-radical power. All the samples in a concentration range 10-100 mg/mL were treated as mentioned in DPPH assay, and the percentage remaining of DPPH was plotted against the concentration to calculate median effective concentration (EC_{50}) and antiradical power ($1/EC_{50}$) as described by Brand-Williams *et al.* [23].

Antioxidant Activity by β-carotene Linoleate Model: All the sample and the standard solutions were prepared as mentioned in DPPH model using ethanol as a solvent.

The assay was performed as described by Taga *et al.* [25], briefly, 1 mL of β-carotene (0.2 mg/mL in chloroform), 20 mg linoleic acid and 200 mg tween 40 (polyoxyethylene sorbitane monoleate) were mixed in a round bottom flask and chloroform was removed by rotary evaporator at 40 ^{o}C. The residue was mixed with 10 mL distilled water and volume was made 50 mL with oxygenated water. Aliquots (3 mL) of this emulsion were transferred into test tubes containing 200 µL of the sample/standard solutions. A control was prepared by mixing 200 µL of ethanol and 3 mL of the emulsion. A blank was prepared by mixing 200 µL of ethanol and 3 mL of the emulsion, without having β-carotene. All the test tubes were incubated in water bath at 50^{o}C and the absorbance was measured at 470 nm against the blank at 0 min and then after every 15 min until color of β-carotene in the control disappeared (120 min). The degradation rate (R) of β-carotene was calculated using the following equation.

$$R = \ln (a/b) X 1/t$$

The terms of the equation are as "ln" (natural log), "a" (absorbance at 0 min), "b" (absorbance at 120 min) and "t" (time in min).

Percentage antioxidant activity (%AA) was calculated using the following equation.

$$\% AA = 100*[(R_{control} - R_{sample})/R_{control}]*1/120]$$

In Vivo Biological Studies of Lab-Made Kanji

The study was conducted as per approved protocol of the Human Research Review Board of University College of Pharmacy, University of the Punjab, Lahore, Pakistan. Written consent was acquired from all the participants. Six, consented, adult healthy male volunteers, aged (21.6 ± 1.14 years) and weight (78.40 ± 8.68 Kg) were selected for the study. Prior to treatment, the blood of each of the individuals was analyzed at Clinical Laboratory, Health Center, University of the Punjab, Lahore, Pakistan for blood cellular components, liver function markers and serum lipid profile, which served as a baseline. One glass of *Kanji* (240 mL) was given daily at noon to all the individuals for two weeks. No restriction of diet and daily activity was imposed on the volunteers throughout the study period. After two weeks treatment, blood samples of all the subjects

were analyzed in the same laboratory for the above stated parameters.

Statistical Analysis

All the samples were analyzed in triplicate and results were presented as mean ± Standard deviation. The data were analyzed using one way ANOVA with Post Hoc multiple comparison Bonferroni. A P value less than 0.05 were taken as significant.

Conclusion

Kanji is a beneficial and safe antioxidant remedy for the protection of liver from oxidative stress and hot weather effects. However, there is a need to devise and adopt a standard protocol for its preparation.

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