Evaluation of antioxidant and antigenotoxic effects of *kahwa*

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*Kahwa* is an aromatic, refreshing and stimulant traditional drink made of various constituents like saffron, cardamom, cinnamon, clove, liquorice and green tea leaves. The people of Kashmir use several forms of *kahwa* depending upon the ingredients added, which may have different health benefits. This is the first study to demonstrate the antioxidant and antigenotoxic potential of one of the common forms of *kahwa* consumed in Baramulla district of Jammu & Kashmir (J&K). The ingredients used for the preparation of this *kahwa* are saffron, cinnamon and green cardamom. The antioxidant and antigenotoxic effects of *kahwa* was evaluated by using various *in vitro* models like DPPH, ABTS, NO, lipid peroxidation, hemolysis and alkaline comet assay. The total phenol and flavonoid content was also determined by using gallic acid and quercetin as a standard. The IC$_{50}$ values of *kahwa* for antioxidant assays, such as DPPH, ABTS, NO scavenging and anti lipid peroxidation were 2.15, 4.8, 8.4 and 0.56 mg/ml, respectively. *Kahwa* was found to have an appreciable amount of polyphenols (7.41 mg GAE/gm) and flavonoids (1.39 mg QE/gm), which may be responsible for its biological activity. Moreover, it was observed that *kahwa* ameliorates H$_2$O$_2$-induced hemolysis and DNA damage. These findings indicated promising antioxidant and antigenotoxic potential of *kahwa* and further studies are required to explore its health benefits.

**Keywords:** Antioxidant, Antigenotoxic, Comet assay, Kahwa, Flavonoids, Polyphenols

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*Kahwa* is a traditional drink consumed in the Kashmir region of India, northern Pakistan, some regions of Central Asia and Afghanistan. It is prepared from various constituents like saffron (*Crocus sativus*), green cardamom (*Elettaria cardamomum*), cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), liquorice (*Glycyrrhiza glabra*) and green tea (*Camellia sinensis*), which have well documented benefits. *Kahwa* is usually of various types and named according to the type of ingredient used in it. *Doodh Kahwa*, the main ingredient is milk, *Dalchine Kahwa*, main ingredient cinnamon, *Teth Kahwa*, main ingredient green tea, *Kung kahwa*, main ingredient saffron, *Lemb Kahwa*, main ingredient lemon and *Shangri Kahwa* main ingredient Liquorice, are some of the different types of *kahwa*. It is traditionally used for treating headaches, digestive and cardiac diseases. The ingredients used in the preparation of the *kahwa* commonly consumed in district Baramulla of J&K are saffron, cinnamon and green cardamom. *Crocus sativus* common name saffron belongs to family Iridaceae, is a perennial plant that is found in various regions of the world. In traditional medicine saffron is used as an antidepressant, respiratory drug, sedative and carminative. Genoprotective, chemopreventive and antioxidant effects of saffron extract were observed in various studies conducted on animal models. *E. cardamomum*, is a herbaceous perennial plant of the family Zingiberaceae. It is known as "*Chhoti ilaichi*" in Unani system of medicine. Seeds of cardamom are widely used for flavoring of food. Its antioxidant activity is because of its constituents, kaempferol, quercetin, luteolin and pelargonidin. *Cinnamomum zeylanicum*, is a small, tropical and evergreen plant, from the family Lauraceae. Cinnamon has a long history as it is used as a spice and flavoring agents since from ancient times. Cinnamon is one of the commonly used spices in the world. The antioxidant property of cinnamon was attributed to its active compounds such as camphene, phenol, epicatechin and tannins. *Cinnamomum cassia* showed protection against H$_2$O$_2$-induced oxidative DNA damage in lymphocytes.

In this study, antioxidant, antigenotoxic potential and phytochemical composition of one of the common forms of *kahwa*, a polyherbal drink consumed in District Baramulla of J&K was investigated.

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Materials and methods

Plant materials
The identification of plant material was done by Dr Arbeen A Bhat, Taxonomist, Department of Botany, Lovely Professional University, Phagwara, India. The plant parts used in this study was *Crocus sativus* L. (saffron) stigma (Voucher no. LPUDP-1021), *Elettaria cardamomum* Maton (green cardamom) fruit (Voucher no. LPUDP-1022) and *Cinnamomum zeylanicum* Breyn. (cinnamon) bark (Voucher no. LPUDP-1023) were purchased from the local market of Sopore, District Baramulla, J&K, India.

Chemicals and reagents
Methanol, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid) (ABTS), Sodium persulphate, Sodium nitroprusside, Sulphanilamide, O-phosphoric acid, Napthylethylene diamine dihydrochloride (NEDA), Ferrous sulphate (FeSO₄), Thiobarbituric acid (TBA), Tri-choloro acetic acid (TCA), Hydrochloric acid (HCL), Butylated hydroxytoluene (BHT), Sodium carbonate (Na₂CO₃), Folin-Ciocalteu’s reagent. All chemicals were purchased from reputed companies.

Sample preparation
*Kahwa* was prepared in a traditional way. Four pieces of cinnamon (3.9 gm), four green cardamoms (1.26 gm) and four strands of saffron (0.008 gm) were added into 100ml of boiling water. Then allowed to boil for 5 min. Keep stirring with the help of a stirrer and cover it for 2 min (Ezinearticles.com/kashmiri-kahwa-Tea). Now, pour it in a beaker and allow it to cool at room temperature. Filter it by using Whatman’s filter paper and the filtrate was used for further investigations.

Antioxidant activity

**DPPH radical scavenging assay**

The radical scavenging activity of *kahwa* against stable DPPH was determined spectrophotometrically. DPPH solution (0.3 mM) was prepared by dissolving 11 mg of DPPH in 50 ml of methanol. The optical density (OD) of DPPH solution was set between 0.8-1 by diluting it with 50 % methanol. Different concentrations of *kahwa* were added separately to 2 ml of DPPH. After 30 min of incubation, the discoloration of the purple to yellow color was measured at 520 nm. Methanol was taken as blank and 2 ml of DPPH solution was taken as control. The experiment was carried out in triplicate.

DPPH Radical scavenging activity % = \( \frac{A_{520}(c) - A_{520}(s)}{A_{520}(c)} \times 100 \)

Where, \( A(c) \) = absorbance of control and \( A(s) \) = absorbance of sample.

**ABTS radical scavenging assay**

This assay is based on the ability of different substances to scavenge 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid) ABTS in comparison to a standard (ascorbic acid). The radical cation was prepared by mixing 7 mM ABTS stock solution (36 mg of ABTS in 100ml of distilled water) with 2.4 mM potassium persulfate (57 mg of sodium persulphate in 100 ml of distilled water) in 1: 1 ratio. Then placed the mixture for 16 hrs in the dark at room temperature until the reaction is complete. The optical density (OD) of ABTS solution was set between 0.8–1.0) by diluting it with 50 % methanol. Different concentrations of *kahwa* were added to every 2 ml of ABTS solution. After 30 min of incubation, absorbance of respective samples was taken at 745 nm.

ABTS Radical scavenging activity % = \( \frac{A_{520}(c) - A_{520}(s)}{A_{520}(c)} \times 100 \)

Where, \( A(c) \) = absorbance of control and \( A(s) \) = absorbance of sample.

**Nitric oxide scavenging assay**

Sodium nitroprusside (10 mM) in 0.9 ml of the phosphate buffer solution was incubated at different concentration (100-400 µg/ml) of *kahwa*, dissolved in phosphate buffer (1M, pH 7.4) and the tubes were incubated at 37 °C for 30 min. After incubation, 0.5 ml Griess reagent (1 % sulphanilamide, 2 % O-phosphoric acid & 0.1 %NEDA) was added. The absorbance was taken at 546 nm. Phosphate buffered saline will be used as blank in this case.

% Scavenging activity = \( \frac{A_{546}(c) - A_{546}(s)}{A_{546}(c)} \times 100 \)

Where, \( A(c) \) = absorbance of control and \( A(s) \) = absorbance of sample.

**Lipid peroxidation (LPO) assay**

Fresh chicken liver was collected from slaughter house, kept in cold or chilled PBS and maintained at
4 °C till use. Thin slices were cut using a sterile scalpel. 10 % liver homogenate was prepared and the supernatant was taken for lipid peroxidation (LPO) estimation. Various concentrations of kahwa were added to every 0.5 ml of liver homogenate. Appropriate controls were set. The mixture of 0.5 ml of liver homogenate, different concentrations of kahwa and 100 µl of FeSO₄ was incubated at 37 °C for 30 min. After incubation, 2 ml of TBA, TCA and HCl in (1:1:1) ratio were added to this mixture and was kept in a water bath for 20 min at 100 °C. Then cooled, centrifuge and the absorbance of supernatant were determined at 532 nm.

The percentage inhibition of LPO = A₅₃₂ (c) – A₅₃₂ (s)/A₅₃₂ (c)
Where, A(c) = absorbance of control and A(s) = absorbance of sample.

**Determination of total polyphenols**

The total polyphenol content (TPC) was determined by using gallic acid as standard. The kahwa samples were mixed with 0.5 ml of 0.2 N Folin-ciocalteau reagents. After 5 min 2.0 ml of 20 % sodium carbonate was added. The absorbance of reaction was measured at 765 nm after 30 min of incubation at 37 °C. The standard curve was prepared by 20-100 µg/ml solution of Gallic acid in methanol. The total phenolic content was measured in reference to gallic acid curve.

**Total flavonoid assay**

Total flavonoid content was measured by the colorimetric Aluminum chloride method. 0.5ml of kahwa was mixed with 1.5 ml of methanol, 0.1ml of 10 % aluminum chloride, 0.1ml of 1 M potassium acetate, and 2.8 ml of distilled water. The solution was incubated at 37 °C for 30 min. The absorbance of the mixture was measured at 415 nm. Total flavonoid contents were calculated from the curve obtained from quercetin, which was taken as the standard. The standard curve was prepared by 10 to 40 µg/ml concentration of quercetin in methanol.

**Erythrocyte haemolysis**

Blood samples were collected from an adult male and female of age group 23-26 yrs. in a heparin coated tube. The study was approved by institutional research committee and also informed written consent were taken from blood donors. The collected blood was centrifuged for 5 min at 1500 rpm. Remove the buffy and the plasma layer to eliminate leukocytes and platelets. Erythrocytes were washed three times with cold phosphate saline (pH 7.4) and centrifuged for 5 min at 1500 rpm. A portion of 200 µl erythrocytes was delivered into a tube and different concentration of treatment into different tubes followed by 20 µl of hydrogen peroxide in order to induce haemolysis and incubate it for 3 hrs at 37 °C. After incubation, add 2 ml of phosphate buffer saline into each tube and centrifuge these tubes at 5000 rpm for 10 min. The absorbance was measured at 540 nm. The increase in absorbance indicates greater haemolysis.

**Alkaline comet assay**

The alkaline comet assay is used to determine single strand breaks in DNA. The viability of lymphocytes were checked by using trypan blue dye exclusion method was performed on isolated lymphocytes. The slides for precoating should be grease free and clean. The slides were dipped in a beaker of melted 1 % normal agarose in water. Let the slides dry for overnight. Mix 50 µl of cell suspension and 150 µl low melting agrose. Spread the suspension on each slide precoated with 1 % normal agrose and covered the slides with cover slips and kept these slides in a refrigerator for 10 min. Remove the coverslips and incubated it for 2 hrs in a lysis solution containing 1 % SDS (sodium dodecyl sulfate), 2.5 M NaCl, 0.1M EDTA, 10 mM Tris and 10 ml Triton X- 100 at 4 °C, add NaOH to ensure that EDTA dissolves and check pH 10. Then put these slides in an electrophoresis tank that was filled with pretreated electrophoretic buffer, (1mM EDTA and 0.3 M NaOH) and incubated it for 20 min. At 25 V, electrophoresis were done in 20 min by using a power supply. When the electrophoresis was complete, washed the slides with PBS (pH 7.5), water and then with ethanol. Then, the slides were stained with ethidium bromide (20 µg/ ml) and observed under fluorescent microscope. The comet tail length was measured by using the Comet Score (version 1.5).

**Statistical analysis**

The data were expressed as mean ± SD for triplicate readings. The statistical comparisons are performed by one way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference test using SPSS software (version 18). If the p-values are 0.05 or less, the results will be considered statistically significant.
Results

DPPH radical scavenging activity
DPPH is a stable free radical that can be reduced by the antioxidants. The percentage scavenging effect on DPPH radical was increased with the increase in concentration of kahwa from 1.275 – 3.825 mg/ml ($P \leq 0.05$), (Fig. 1). The IC$_{50}$ value of the kahwa was calculated to be 2.15 mg/ml, while for the standard (ascorbic acid) is 0.0046 mg/ml.

ABTS radical scavenging activity
The antioxidant capacity of kahwa was evaluated according to the ABTS decolorization method. The percentage of the scavenging effect on ABTS radical was increased with the increase in the concentration of kahwa from 1.275 – 5.1 mg/ml ($P \leq 0.05$), (Fig. 2). The IC$_{50}$ value of the kahwa was calculated to be as 4.8 mg/ml and for standard (ascorbic acid), it was found to be 0.0025 mg/ml.

Nitric oxide radical scavenging activity
The percentage scavenging activity of nitric oxide radical was increased with the increase in concentration from 5.1–20.4 mg/ml (Fig. 3). A significant increase in scavenging activity ($P \leq 0.05$) of kahwa was observed from 34.55 % at 5.1 mg/ml to 64.17 % at 15.3 mg/ml, no statistically significant change was found in scavenging activity with further increase in concentration from 15.3 to 20.4 mg/ml. The IC$_{50}$ of kahwa was calculated to be 8.4 mg/ml and for standard (ascorbic acid), it was found to be .0026 mg/ml.

Lipid peroxidation assay
The percentage scavenging activity was increased with increase in dose from 0.51 to 2.04 mg/ml (Fig. 4). Initially at 0.51 to 1.02 mg/ml there was no significant increase in the scavenging activity, then from concentration 1.02 to 1.53 mg/ml there was statistically significant difference ($P \leq 0.05$) in the scavenging activity and from 1.53 to 2.04 mg/ml no significant difference was observed. The IC$_{50}$ of the kahwa was calculated to be 0.56 mg/ml and for the standard (BHT) it was calculated to be .0062 mg/ml.

Fig. 1- DPPH radical scavenging activity of kahwa (mean ± sd, n=3). Different lower case alphabets (a-c) indicate statistically significant difference at $P \leq 0.05$ by Tukey’s test.

Fig. 2- ABTS radical scavenging activity of kahwa (mean ± sd, n=3). Different lower case alphabets (a-d) indicate statistically significant difference at $P \leq 0.05$ by Tukey’s test.

Fig. 3- Nitric oxide radical scavenging activity of kahwa (mean ± sd, n =3). Different lower case alphabets (a-c) indicate statistically significant difference at $P \leq 0.05$ by Tukey’s test.

Fig. 4- Anti-Lipid peroxidation activity of kahwa (mean ± sd, n = 3). Different lower case alphabets (a-b) indicate statistically significant difference at $P \leq 0.05$ by Tukey’s test.
Total phenolic content (TPC)
The therapeutic effects obtained from many medicinal plants have been ascribed to the presence of phenolic compounds. A linear calibration curve of gallic acid in the range of 40–200 µg/ml, with a coefficient of determination (R²) value of 0.983 was obtained (Fig. 5). The total phenolic content is represented as gallic acid equivalents. The total phenolic content in the kahwa was measured to be 7.41± 2.35 mg/g.

Total flavonoid content
Flavonoids are polyphenolic compounds, which exhibit several biological effects. The total flavonoid content reported as quercetin equivalent/ml of extract by reference standard curve (Fig. 6). The total flavonoid content in the kahwa was measured to be 1.39 ± 0.019 mg/g.

Erythrocyte hemolysis
The effect of kahwa on hydrogen peroxide induced erythrocyte hemolysis was evaluated. H₂O₂ caused considerable erythrocyte lysis that was significantly inhibited by kahwa. The erythrocyte hemolysis decreases with an increase in the concentration from 0.25 to 0.76 mg/ml. From concentration 0.25 to 0.51 mg/ml there was a significant decrease (P ≤ 0.05) in erythrocyte hemolysis, then from concentration 0.51 to 0.76 mg/ml, no significant difference was observed (Fig. 7).

Alkaline comet assay
In the present study it was observed that treatment of H₂O₂ to lymphocytes induces damage in DNA measured as % DNA in the tail. The pretreatment of cells with kahwa showed a significant reduction in the genotoxicity of H₂O₂ (P ≤ 0.05). The % DNA damage in tail decrease with increase in concentration of kahwa extract from 14.32 % at 0.255 mg/ml (KE255) to 8.53 % at 0.510 mg/ml (KE510) when compared with 24.01 % in case of H₂O₂ [(+) control] treated cells (Fig. 8).

Fig. 5- Standard calibration curve of total phenolic content.

Fig. 7- Effect of kahwa on H₂O₂-induced erythrocyte hemolysis (mean ± sd, n = 3). Different lower case alphabets (a-b) indicate statistically significant difference at P < 0.05 by Tukey’s test.

Fig. 8- Effect of kahwa on H₂O₂-induced DNA damage to lymphocytes. Different lower case alphabets (a-d) indicate statistically significant difference at P ≤ 0.05 by Tukey’s test. (-) control: only PBS treated lymphocytes; (+) control: Only H₂O₂ treated lymphocytes; KE255: 0.255mg/ml kahwa plus H₂O₂ treated lymphocytes; KE510: 0.510mg/ml kahwa plus H₂O₂ treated lymphocytes.
Discussion

It is well known that free radicals play an important role in various pathological diseases. Antioxidants defend us from various diseases by scavenging these free radicals. They perform this activity in two ways, either by reducing the reactive oxygen species or by defending the endogenous antioxidant defense mechanisms. The type of kahwa used in this study consists of green cardamom, saffron and cinnamon. The DPPH scavenging activity of methanolic extract of E. cardamomum showed an IC\text{50} value of 0.681 mg/ml\textsuperscript{22}. A dose dependent increase was observed in scavenging activity of ethanolic leaf extract of Cinnamomum zeylanica against DPPH free radical. At 0.1 mg/ml the highest DPPH radical scavenging activity (92.1 ± 0.06) was observed in C. zeylanica higher than the BHT (85 ± 1.1 %) and the other species of cinnamon\textsuperscript{23}. In the present study, Kahwa demonstrated DPPH radical scavenging activity of 29.07 % at 1.275 mg/ml to 69.82 % at 3.825 mg/ml. It was reported that the IC\text{50} value for methanolic extract of C.sativus flower was 0.202 mg/ml for ABTS and for ascorbic acid it was 0.017 mg/ml\textsuperscript{24}. In current work, kahwa showed the IC\text{50} of 4.8 mg/ml and for standard (ascorbic acid), it was calculated to be 0.0025 mg/ml. In the present study, kahwa demonstrated ABTS radical scavenging activity of 11.97 % at 1.275 mg/ml to 51.76 % at 5.10 mg/ml.

Nitric oxide is a free radical and it plays an important role in the regulation of various physiological processes in the cells of mammals. When these radicals are produced in excess they may lead to various diseases\textsuperscript{25}. The reduction of NO radical by cardamom seeds was found to be higher in aqueous extract than in methanolic extract. The methanolic extract of E. cardamomum showed 9.35 ± 0.31 % scavenging activity while the aqueous extract showed 12.05 ± 0.75 % \textsuperscript{26}. Previous reports showed lower IC\text{50} or higher antioxidant activity of saffron, cardamom and cinnamon compared to kahwa in the current study, this is because decoction of kahwa as consumed by the general public is being evaluated in the present investigation. The total phenolic content in the kahwa was measured to be 7.41 ± 2.35 mg/gm. The total phenolic and flavonoid content in the methanolic extract of C. sativus was found to be 86.65 mg/gm\textsuperscript{21}, and that of 80 % aqueous-methanolic extract of E. cardamomum was 2.85 ± 0.23 and 1.83 ± 0.40 mg/gm, respectively\textsuperscript{27}. Earlier reports showed that not only the presence of high concentration of polyunsaturated fatty acids in erythrocyte membranes, but also the active oxygen transport by hemoglobin makes the erythrocytes susceptible to oxidative stress. The potential of kahwa to inhibit haemolysis may be due to its potential to scavenge the ROS produced during metabolism of H\textsubscript{2}O\textsubscript{2} in the living cell. In current investigation Kahwa showed inhibition of erythrocyte hemolysis from 79.18 % at 0.25 mg/ml to 90.34 % at 0.76 mg/ml. The present study by employing standard alkaline comet assay revealed that H\textsubscript{2}O\textsubscript{2} treatment induces an increment in DNA migration, while the extent of damage was reduced in case of pretreatment with kahwa. The antigenotoxic potential of kahwa may be due to its free radical scavenging ability, that help in neutralizing radicals formed by the metabolism of H\textsubscript{2}O\textsubscript{2} in biological system. The genoprotective effect of C. sativus stigma extract (CSE) and crocin (trans-crocin 4) on methyl methanesulfonate (MMS) –induce the DNA damage in different organs of the mice was observed. The genoprotective effect of crocin and saffron may be observed due to its antioxidant and free radical scavenging properties\textsuperscript{28}.

Conclusion

Kahwa a traditional polyherbal drink, can scavenge free radicals and may prevent us from many diseases such as neurodegenerative, cardiovascular, arthritis, diabetes and cancer, etc. It showed antioxidant property that may be ascribed to its phytochemical composition, especially polyphenols and flavonoids that are believed to play important role in radical scavenging activity. Moreover, it was observed that kahwa ameliorates H\textsubscript{2}O\textsubscript{2}-induced haemolysis and DNA damage. Kahwa was also found to have an appreciable amount of polyphenols and flavonoids, which may be responsible for its biological activities. These findings indicated promising antioxidant and antigenotoxic potential of kahwa extract and needs further exploration for its health benefits.

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