Phytochemical screening and HPLC-UV method quantification of Flavonoids in *Coffee Arabica* green seeds

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**ABSTRACT**

The green coffee bean is the most commonly used beverages in India and it is one of the most commercialized food products. They have a rich source of biologically active compounds that are important for human health. The coffee tree or a shrub belongs to the family *Rubiaceae*. Commercially available, two species of green coffee bean are *Coffea Arabica* and *Coffea canephora*. Cancer is the most important cause of death. Apart from cancer, quercetin can also prevent Osteoporosis. The phytochemicals present in the green coffee bean can be used as an alternate therapy for cancer due to its antimitotic activity and free radical scavenging activity. Total antioxidant shows IC\textsubscript{50} value 45.81. Kaempferol is a potent antioxidant that can defend against free radicals and cure chronic diseases. Flavonoids are phenolic substances that act as an antioxidant, anti-inflammatory, anti-allergenic, antiviral and also have vasodilating actions. Green coffee bean shows a high concentration of Flavonoids in hydroethanolic extraction. The aim of this study is used to analyse the presence of Flavonoids in green coffee bean by using High-performance Liquid Chromatography (HPLC). Flavonoids are potent antioxidant that can bind to a protein. Flavonoids show a wide range of biological and pharmacological activities like anti-allergic, anti-inflammatory, anti-cancer and anti-microbial activity.

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Caffeine is more effective in weight loss and reduce the abdominal and liver fat accumulation by inhibition of macrophages into adipose tissue (Samadi and Mohammadshahi, 2015).

Cancer is the most cause of death. Treatment of cancer involves chemotherapy and surgery but these therapies have side effects with a low cure rate (Li et al., 2016). Along with the chemotherapy, Phytochemicals from the plants can also cure cancer and used as an alternate therapy due to its antioxidant and free radical scavenging activity (Varadharaj and Muniyappan, 2017). To a great extent, the consumption of coffee may reduce colorectal cancer. It may also reduce the influence of Hepatocellular Carcinoma (Akinyemi et al., 2005).

**Morphology**

Coffee is economically important and traded in the market as green coffee. It has a glomerule with bisexual flowers and white petite inflorescence. The coffee bean is surrounded smooth exocarp, fibrous endocarp and a pulpy mesocarp as shown in Figure 1. Exocarp colour can be changed from yellow to red during the last stage. Mesocarp contains water, reducing sugar and sucrose and the digestive enzymes can be protected by the endocarp (Castro and Marraccini, 2006).

Epicarp is the outer layer or the skin of green coffee bean. During depulping, the epicarp is removed and during wash mucilage is also removed (Muñoz et al., 2019).

**Bioconstituents and Phytoconstituents in Green Coffee Bean**

Green coffee bean has both volatile and non-volatile compounds. When it gets roasted, coffee bean compounds gives aroma flavour. Caffeine (1,3,7-trimethyl-xanthine) is the nonvolatile alkaloid present in the green coffee bean. The other non-volatile compounds found in green coffee bean in lower amount are theobromine, methylxanthine, theophylline, libetine and paraxanthine (Clifford and Kazi, 1987). Volatile compounds present in the green coffee bean are aromatic molecules and their derivatives like pyrazine, aldehydes and short-chain fatty acids. These compounds reduce the pleasing odour in green coffee bean rather than the roasted bean (Yeretzian et al., 2002).

On maturation, the proteins present in green coffee bean degraded to amino acids. The enzyme catalase is essential for maturation. In Coffea Arabica, the concentration of alanine is high than the asparagine, whereas in Coffea Robusta, the concentration of alanine is less than the asparagine. Cellulose galactomannan and arabinogalactans are the polysaccharides predominantly present in green coffee bean (Murkovic and Derler, 2006).

**Phytoconstituents of Coffee arabica**

Polyphenols can prevent oxidative stress, all different types of cancer and cardiovascular disease and this compound present in beverages, as well as in foods. In coffee, the more abundant polyphenolic constituent are 3-, 4- and 5-Caffeoylquinic acids. Polyphenolic compound plays an important role in defence against pathogens (Matsunaga et al., 2002). Flavonoids bind to the protein and they are potent antioxidant (Arts et al., 2002). Major polyphenols from green coffee beans are hydroxycinnamic acids and quinic acid, together known as chlorogenic acids. Tannins, anthocyanins and lignans are seen in seeds in fewer amounts. Polyphenols from coffee are excellent antioxidants and possess free radical scavenging properties (Yashin et al., 2013).

**MATERIALS AND METHODS**

**Phytochemical Analysis of Coffee arabica**

**Preparation of plant extract**

Extraction of 5 g of dry powdered plant material was taken in 100 ml of 70% of ethanol for maceration periods (24 hr). At room temperature, the extraction was carried out with agitation at 150 rpm. The soaked powder-solvent mixtures were filtered through a Whatman filter paper No. 1, after the maceration periods. A Part of the filtrate is used for phytochemical analysis and remaining solvent was evaporated to dry for in vitro antioxidant studies.

**Qualitative analysis**

Preliminary phytochemical analysis was carried out by using standard procedure Sofowara (1993) and Harborne (1973).

**Quantitative analysis**

The amounts of total phenolic contents of plant were determined by the spectrophotometric method of Kim et al. (2003) with slight modification. The total flavonoids assay was conducted according to Damodor et al. (2011). Total flavonoids content was determined by using Aluminium chloride colorimetric method. The total Tannins assay was conducted according to Bajaj and Devsharma (1977) method.

**In vitro Anti-oxidant activity**

**DPPH radical-scavenging activity**

DPPH radical-scavenging activity was determined by the method of Shimada et al. (1992). The antioxidant activity of the extracts was evaluated by the...
phosphomolybdenum method according to the procedure of Prieto et al. (1999). The superoxide anion radicals scavenging activity was measured by the method of Liu et al. (1997). The scavenging activity for hydroxyl radicals was measured with the Fenton reaction by the method of Yu et al. (2004). Nitric oxide radical scavenging activity was determined according to the method reported by Garrat (1964).

RESULTS AND DISCUSSION

Qualitative Analysis

By the qualitative analysis, Coffee arabica shows the presence of phytochemical constituents, which include Glycoside, Tannin, Saponin, Polypheno, Anthocyanin, Flavonoids, Phenol, Triterpenoid, Coumarins, Anthroquinone, Alkaloids as shown in Table 1.

Figure 1: Cross-section of Green Coffee Bean

Figure 2: Phytochemicals analysis in Coffee arabica

Figure 3: Standard Curve for Flavonoids using Quercetin

Figure 4: Standard Curve for Phenol using Gallic acid

Figure 5: Standard Curve for tannin using tannic acid

Figure 6: DPPH radical scavenging activity of Green Coffee Bean

Coumarin, Glycoside, flavonoid, Steroid, Terpenoid shows higher concentration in hydroethanolic extract. The human immune system can protect the viral infections more efficiently due to the presence of Terpenoids and it has anti-inflammatory activities.

Coumarin has potential to chelate the metal ions...
Table 1: Phytochemicals analysis in *Coffee arabica*

| S.No. | Test analysis     | Hydro alcoholic |
|-------|-------------------|-----------------|
| 1     | Tannin            | +               |
| 2     | Phlobatannin      | -               |
| 3     | Saponin           | +               |
| 4     | Flavonoids        | ++              |
| 5     | Steroids          | ++              |
| 6     | Terpenoids        | ++              |
| 7     | Triterpenoids     | +               |
| 8     | Alkaloid          | -               |
| 9     | Anthroquinone     | ++              |
| 10    | Polyphenol        | +               |
| 11    | Glycoside         | ++              |
| 12    | Coumarins         | ++              |
| 13    | Emodins           | -               |
| 14    | Anthocyanins      | -               |

(-) Absent, (+) Present and (++) high concentration

Table 2: Quantitative analysis of phenol, flavonoids and tannin content of *Coffee arabica*

| Sample         | Total phenol (Milligrams of Gallic acid (GAE) equivalents per gram) | Flavonoids (Milligrams of quercetin equivalents per gram) | Tannin (Milligrams of tannic acid equivalents per gram) |
|----------------|------------------------------------------------------------------|----------------------------------------------------------|--------------------------------------------------------|
| *Coffee arabica* | 205.02 ±14.35                                                    | 121.38 ± 8.49                                            | 61.04 ± 4.27                                           |

Values are expressed as Mean ± SD for triplicates

Table 3: DPPH radical scavenging activity of *Coffee Arabica* green seeds

| Samples                      | Concentrations (µg/ml) | IC50 Value |
|------------------------------|------------------------|------------|
|                              | 20                     | 40         | 60         | 80         |
| *Coffee arabica* green seeds| 23.64±1.65             | 47.73±3.34 | 71.37±4.99 | 87.28±6.10 | 43.01      |
| Ascorbic acid (Std.) (% of inhibition) | 26.54±0.95             | 38.6±2.04  | 79.26±4.90 | 96.98±7.11 | 41.70      |

Values are expressed as Mean ± SD for triplicates

Table 4: Total antioxidant activity of *Coffee arabica*

| Samples                      | Concentrations (µg/ml) | IC50 Value |
|------------------------------|------------------------|------------|
|                              | 20                     | 40         | 60         | 80         |
| *Coffee Arabica* green seeds| 21.56±1.50             | 44.68±3.12 | 67.81±4.74 | 83.43±5.84 | 45.81      |
| Ascorbic acid (Std.) (% of inhibition) | 22.35±1.80             | 43.67±2.61 | 51.23±4.09 | 72.54±5.80 | 53.22      |

Values are expressed as Mean ± SD for triplicates
Table 5: Superoxide anion radical scavenging activity of *Coffee arabica* green seeds

| Samples                  | Concentrations (µg/ml) | IC<sub>50</sub> Value |
|--------------------------|------------------------|-----------------------|
| *Coffee arabica* green seeds (%) of inhibition | 20 | 47.14±3.29 | 78.92±5.52 | 45.13 |
| Ascorbic acid (Std.) (%) of inhibition | 20 | 31.25±2.50 | 89.54±7.16 | 34.23 |

Values are expressed as Mean ± SD for triplicates

Table 6: Hydroxyl radical scavenging activity of *Coffee arabica* green seeds

| Samples                  | Concentrations (µg/ml) | IC<sub>50</sub> Value |
|--------------------------|------------------------|-----------------------|
| *Coffee arabica* green seeds (%) of inhibition | 20 | 46.25±3.23 | 81.50±5.70 | 45.88 |
| Ascorbic acid (Std.) (%) of inhibition | 20 | 56.45±4.40 | 92.75±7.2 | 35.26 |

Values are expressed as Mean ± SD for triplicates

Table 7: Nitric oxide scavenging activity of *Coffee arabica* green seeds

| Samples                  | Concentrations (µg/ml) | IC<sub>50</sub> Value |
|--------------------------|------------------------|-----------------------|
| *Coffee arabica* green seeds (%) of inhibition | 20 | 52.38±3.66 | 82.38±5.76 | 42.34 |
| Ascorbic acid (Std.) (%) of inhibition | 20 | 59.62±4.65 | 96.45±7.52 | 35.36 |

Values are expressed as Mean ± SD for triplicates

Table 8: HPLC profile of Green Coffee Bean

| Peak# | Ret. Time | Area | Height | Area % | Height % | Compounds identified by literature** |
|-------|-----------|------|--------|--------|----------|-------------------------------------|
| 1     | 2.287     | 14109| 351    | 0.206  | 0.090    | Kaempferol                           |
| 2     | 3.449     | 2985654| 46174 | 43.359 | 11.869   | Quercetin                            |
| 3     | 7.691     | 3080175| 341910| 55.608 | 87.887   | Hypersoide                           |
| 4     | 13.541    | 40259| 600    | 0.588  | 0.154    | Delphinidin-3-O-Glucoside (Anthocyanin) |
| Total |           | 6848198| 389036| 100.000| 100.000  |                                     |

Flavonoids are visual attractors, photoreceptors, and they also exhibit biological activities, which include antibacterial, antifungal, anti-inflammatory and anti-allergic activities. Flavonoids have an antiaggregatory effect so that they can able to scavenge the superoxide free radicals. Due to the anti-inflammatory effect, it has the ability to reduce the formation of reactive oxygen species, nitric oxide, leukotrienes, prostaglandins (Robak and Gryglewski, 1996).

Quantitative analysis

Quantitative analysis of Total tannin, Total phenol and Total flavonoids were determined on a hydroethanolic extract of *Coffee Arabica*. Flavonoid and phenol show higher concentration. The total flavonoid content was found to be 121.38 ± 8.49, which was shown in Table 2 and was predicted in Figure 3. Flavonoids have an antithrombotic effect and hence it has ability to scavenge the free radicals. Steroids have a relationship with sex hormones because they have importance in pharmacy (Savithramma et al., 2011). Terpenoids and triterpenoids play an important role in the human immune system, which has the ability to scavenge free radicals (Grassmann, 2005).

Flavonoids have a relationship with sex hormones because they have importance in pharmacy (Savithramma et al., 2011). Terpenoids and triterpenoids play an important role in the human immune system, which has the ability to scavenge free radicals (Grassmann, 2005).
and also show a high effect on coronary artery disease (Castelnuovo et al., 2012). Linoleic acid shows more effect against flavonoids which may show scavenging activity on peroxyl radical (Torel et al., 1986). Flavonoid shows antiradical activity depending upon the hydroxyl group at the C4 position. They show some protective effect like vasorelaxant and anti-ischemic activity. Flavonoids inhibit the formation of cancer from procarcinogen. They also play an important role in the brain and degenerative diseases (Benavente-García and Castillo, 2008).

The total phenol content and its graphical representation were given in Table 2, Figure 4. The pharmacological action of phenolic compounds acts against oxidative pressure which may include antiulcer, antiplasmodic, antidepressant and cytotoxic activities.

Phenolic compounds show chemopreventive properties like antimutagenic, anti-inflammatory and antioxidant. They inhibit the oncogenesis process and block the cell signalling pathway (Huang et al., 2009).

The total tannin content was 61.04 ± 4.27, as shown in Table 2 and it was predicted in Figure 5. Tannin has an anti-inflammatory effect and a good healing capacity. They also involve in the treatment of piles and inflammation (de Sousa Araújo et al., 2008). Tannin plays an important role in protecting against chronic diseases (Raoof et al., 2017).

DPPH radical scavenging activity

DPPH radical scavenging activity was determined and the IC₅₀ value of coffee Arabica was found to be 43.01, which are near to the standard ascorbic acid, shown in Table 3, Figure 6. Among all the varieties of green coffee bean only coffee Arabica show the highest DPPH radical scavenging activity. It shows high antioxidant activity, low caffeine content and hence it has a wide application in the nutraceutical
industry (Babova et al., 2016). Roasted Coffee Arabica greatly reduces the antioxidant activity, while the extraction of green coffee bean seeds shows high antioxidant activity. In robusta coffee, the caffeine content is high, and hence it shows high antioxidant activity. (Vignoli et al., 2011).

Green seed Coffee Arabica with different concentrations shows IC\textsubscript{50} value 45.81, which was shown in Table 4, and Figure 7 is more than the standard ascorbic acid. With a spin trap coffee, solutions act as superoxide scavengers.

Superoxide radical scavenging activity IC\textsubscript{50} value was shown in Table 5, and results were interpreted in Figure 8. It shows high antioxidant activity and scavenges free radicals. Due to its scavenging activity, it plays an important role in the cancer treatment of cancer (Goodman et al., 1994).

Hydroxy radical damages the biological system extremely because they are one of the important reactive oxygen species. The inhibitory effect of the green coffee arabica was determined since this radical involves an important process in the biological system, which may include mutagenesis and carcinogenesis. Table 6 shows the IC\textsubscript{50} value of hydroxy radical and Figure 9 shows the graphical representation of hydroxy radical activity.

Nitrite and peroxynitrite anion are the free radicals produced with the excess amount of nitric oxides. Neurons, Macrophages and endothelial cells are mediated by these nitric oxides radical. Since it damages the biological system, especially DNA it involves scavenging activity. The IC\textsubscript{50} value of NO Coffee arabica green seeds in Table 7 and Figure 10 shows the maximum inhibitory effect than the ascorbic acid (Gunalan et al., 2012).

**HPLC Analysis**

**Extraction**

The green coffee beans were washed thoroughly and shade dried at room temperature. Dried coffee beans were made into powder using a mechanical grinder. 3kg of the green coffee beans powder were weighed and soaked in a 30:70 ratio of hydroethanol for 15 days at room temperature with agitation at 150 rpm. After the maceration periods, the soaked powder-solvent mixtures were filtered through a Whatman No. 1 filter paper and the crude extract were lyophilized into a paste and were taken for further investigation.

**Procedure**

Flavonoids were analysed by using an HPLC method (Samee and Vorarat, 2007). The HPLC analysis of Green Coffee Bean was carried out with a Chromatographic system (Shimadzu Class-VPV6.14SP2, Japan) consist of an autosampler with a 20μl fixed loop and a UV-Visible detector. The gradient elution of solvent A [water-acetic acid (25:1 v/v)] and solvent B (methanol) had a significant effect on the resolution of compounds. As a result, solvent gradients were formed using a dual pumping system by varying the proportion of solvent A [water-acetic acid (25:1, v/v)] to solvent B (methanol). Solvent B was increased to 50% in 4 min and subsequently increased to 80% in 10 min at a flow rate of 1.0 mL/min. The samples were run for 25 min. And detection was done at 280 nm by a UV detector (Lamp-D2). All chromatographic data were recorded and processed using autochromatography software and the profile of the sample was shown in Figure 11.

The hydroethanol extraction of green coffee bean on HPLC analysis reveals the presence of Flavonoids and their compounds. Among all the phytochemicals, the concentration of Flavonoid is high in the green coffee bean and shows a high peak in the chromatogram as shown in Figure 11. Table 8 show the compounds identified in the green coffee bean. The major flavonoids present in the green coffee bean are Kaempferol, Quercetin, Hypersoide, Delphinidin-3-O-Glucoside (Anthocyanin).

Kaempferol has anticancer activity, which may reduce the risk of various cancers. In this study, the compound present in the hydroethanolic extract of the green coffee bean is the flavonoids which may include Kaempferol, Quercetin, Hypersoide, Delphinidin-3-O-Glucoside (Anthocyanin). Of these, the Hypersoide compound shows the highest peak value with 87.887% height, as shown in Table 8.

Flavonoids are the phenolic substances and act as an antioxidant, antimicrobial, visual attractors, photoreceptor. They also exhibit biological activities which include anti-inflammatory, anti-allergic, antiviral and also have vasodilating actions (Pietta, 2000). On the HPLC-UV method, by the supercritical CO\textsubscript{2} extract, lower level of chlorogenic acids were obtained and on ethanol extract, a high level is seen (Craig et al., 2016). Microwave-assisted soxhlet extraction of 13 different green coffee bean shows six times higher value of diterpenes than the traditional method (Tsukui et al., 2014). The Green and roasted coffee bean, which belongs to coffee Arabica and Coffee Robusta are extracted by soxhlet with hexane. And this extraction was performed with HPLC analysis and the compounds obtained are tocopherols and triglycerides. A result of this method shows the differentiation between the coffee varieties, especially between green and roasted.
Kaempferol is a potent antioxidant and can be found in both vegetables and fruits. The risk of chronic diseases can be reduced by the kaempferol compound because of its defence against free radicals. Kaempferol also induces apoptosis i.e. programmed cell death pathway and prevent the formation of cancer cells (Chen and Chen, 2013). Apoptosis occurs due to the elevation of oxidative stress because kaempferol induces glioma cells (Sharma et al., 2007).

A natural flavonoid present in the plant species is the kaempferol which has potent inflammatory activities (Devi et al., 2015). Quercetin can prevent Lung cancer due to its anti-oxidative property and also has free radical scavenging activity. Glycoside is the compound present and later, it is converted to quercetin (Murakami et al., 2008). Quercetin has an anticancer activity by inhibiting the tumor growth. It also regulates the cell cycle process by inhibiting the tyrosine kinase activity (Lamson and Brignall, 2000).

Osteoporosis can also be prevented by the compound quercetin, which can able to scavenge highly reactive species like hydroxyl radical. Pulmonary and cardiovascular diseases can also be prevented (Boots et al., 2008). Anthocyanin is a potent antioxidant and involves in scavenging activity (Neill and Gould, 2003). Apart from antioxidant, they also have anti-obesity, neuro-protective, antimicrobial, and anti-tumour activities (Smeriglio et al., 2016).

CONCLUSION

The Present investigation shows that the hydroethanolic extract of green coffee bean shows the presence of phytochemicals such as Tannin, Saponin, Flavonoids, Steroids, Terpenoids, Triterpenoids, Anthroquinone, Polyphenol, Glycoside and coumarins. Of these, Flavonoids shows higher concentration. HPLC-UV analysis gives the phytocompound Flavanoid (Kaempferol, Quercetin, Hypersoide, Delphinidin-3-O-Glucoside). Evidently, flavonoids produce antioxidant activity so this mechanism signifies that the extract of green coffee bean may be useful to prevent oxidative stress and also cure various types of cancers. Flavonoids show a wide range of biological and pharmacological activities like anti-allergic, anti-inflammatory, anti-cancer and anti-microbial activity. In future studies, the exact mode of action of Flavonoids can be studied.

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