TOTAL PHENOLICS AND FLAVONOIDS AND ANTIOXIDANT POTENTIAL OF DRAKSHARISHTA PREPARED BY TRADITIONAL AND MODERN METHODS

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Summary

The objective of the present study was to estimate the total phenolic content as well as flavonoids in Draksharishta:T and Draksharishta:M prepared by traditional and modern methods respectively and in its marketed preparation and also to evaluate the antioxidant activity of these test preparations on two different in vitro antioxidant activity models. Total phenolic content was determined colorimetrically using Folin–Ciocalteu reagent and was found 0.0967 and 0.0961 %w/w gallic acid equivalent in Draksharishta:T and Draksharishta:M respectively. Total flavonoid content was determined by aluminium chloride method and was found 0.01163 and 0.01129 %w/w quercetin equivalent in Draksharishta:T and Draksharishta:M respectively. Super-oxide anion scavenging activity and lipid per-oxidation assay were carried out to evaluate the antioxidant potential of Draksharishta:T and Draksharishta:M. The antioxidant activity of Draksharishta:T and Draksharishta:M was found increased in concentration dependent manner in both the in vitro antioxidant activity models as super-oxide radical scavenging activity and lipid per-oxidation assay. Draksharishta:T and Draksharishta:M showed significant scavenging of super-oxide radical and showed IC50 138.06 and 145.35 µg/ml respectively. Draksharishta:T and Draksharishta:M also inhibited the ferrous sulphate induced lipid per-oxidation in dose dependent manner and showed inhibitory concentration (IC50) 230.03 and 236.11 µg/ml respectively. Marketed Draksharishta also showed a rich concentration of total phenolics and flavonoids and showed dose dependent antioxidant activity in both the models. Thus, the results obtained in this study indicate that Draksharishta:T and Draksharishta:M can be a promising source of natural antioxidant.

Key words: Total phenolics, flavonoids, antioxidant potential, lipid per-oxidation assay, super-oxide scavenging activity, Draksharishta.
Introduction

The traditional medicine all over the world now a days is revealed by an extensive activity of research on different plant species and their therapeutic principles\(^1\). Free radicals of different forms are constantly generated for specific metabolic requirement and quenched by an effective antioxidant network in the body. When the generation of these species exceeds the levels of antioxidant mechanism, it leads to oxidative damage of tissues and biomolecules, eventually leading to disease conditions, especially degenerative diseases\(^3\).

As plants produce a lot of antioxidants to control the oxidative stress caused by sun beams and oxygen, they can represent a source of new compounds with antioxidant activity\(^4\). Some of the non-nutritive antioxidants of plants are phenolic compounds, flavonoids, coumarins, benzylisothiocyanate etc\(^5\). Epidemiological evidence indicates an inverse relationship between the intake of food rich in phenolic compounds and the reduction of certain chronic diseases and coronary heart disease mortality\(^6\).

Draksharishta is a polyherbal hydroalcoholic ayurvedic preparation and is used as blood purifier, in the treatment of anaemia and advised as a choice of remedy in respiratory problems. The chief ingredient of Draksharishta is draksha, fruits of *Vitis vinifera*\(^7\). The composition and properties of fruits of *Vitis vinifera*, have been extensively investigated and it was reported that they contain large amount of phenolic compounds as catechins, epicatechin, quercetin, gallic acid, dimeric, trimeric and tetrameric procyanidins\(^8\). These compounds have many favourable effects on human health such as lowering of human low density lipoproteins, reduction of heart disease and cancer etc\(^9\).

Therefore, we undertook the present investigation to estimate the total phenolic content as well as total flavonoids and to evaluate the antioxidant potential of Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively and its marketed preparation on two different *in vitro* antioxidant activity models as super-oxide radical scavenging activity and lipid per-oxidation assay.

Material and Methods

Preparation of Draksharishta-T

This was prepared by the method as given in The Ayurvedic Formulary of India, Part-I\(^7\). All the ingredients of Draksharishta were procured from local market, Jamnagar while jaggery was procured from local market, Mehsana. Authentication of all the ingredients of Draksharishta was done by Dr. G. D. Bagchi, Scientist, Department of Taxonomy and Pharmacognosy, Central Institute of Medicinal and Aromatic Plants, Lucknow. Prepared herbarium has been deposited in the Central Institute of Medicinal and Aromatic Plants, Lucknow for future reference. Identification of all the individual plant material was done as per The Ayurvedic Pharmacopoeia of India. Quantity of ingredients taken for the preparation of batch size 3.25 l of Draksharishta has been calculated according to the formula as given in The Ayurvedic Formulary of India, Part-I, 2000.

According to this method, dried fruits of *Vitis vinifera* after proper crushing were placed in polished vessel of brass along with prescribed quantity of water (13 l), and
allowed to steep overnight. After overnight steeping, this material was warmed at medium flame until the water for decoction reduced to one fourth of the prescribed quantity (3.25 l), then the heating was stopped and it was filtered through unstarched muslin cloth in cleaned and fumigated vessel and after that jaggery was added and mixed properly. Then the prescribed quantity of coarsely powdered prakshepa dravyas as *Cinnamomum zeylanicum* (stem bark), *Eletteria cardamomum* (seeds), *Cinnamomum tamala* (leaves), *Mesua ferrea* (stamens), *Callicarpa macrophylla* (flowers), *Piper nigrum* (fruits), *Piper longum* (fruits), *Embelia ribes* (fruits) were added and then dhataki flowers (*Woodfordia floribunda*) were added for inducing fermentation and after that this sweet filtered fluid was placed for fermentation in incubator for fifteen days at 33ºC±1 ºC. After fifteen days completion of fermentation was confirmed by standard tests$^{13}$. The fermented preparation was filtered with unstarched muslin cloth and kept in cleaned covered vessel for further next seven days. Then, it was poured in clean amber coloured glass bottles previously rinsed with ethyl alcohol, packed and labelled properly.

**Preparation of Draksharishta-M**

Method of preparation of Draksharishta-M was same as followed for Draksharishta-T only dhataki flowers were replaced by yeast for inducing fermentation$^{14}$.  

**Chemicals**

Folin-Ciocalteu and thiobarbituric acid was obtained from Loba Chemie, India. Nitroblue Tetrazolium (NBT) and gallic acid were obtained from Sigma chemicals, St. Louis, USA. Quercetin was purchased from Yucca Enterprises, Bombay. Ferrous sulphate, trichloroacetic acid, potassium dihydrogen phosphate, phenazine methosulphate, sodium carbonate, aluminium chloride, ethanol and methanol etc were of analytical grade and obtained from Ranbaxy Fine Chemicals.

**Estimation of Total Phenolic Content**

Total phenolic content was determined in both types of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively and in its marketed preparation by using Folin Ciocalteu’s reagent$^{15}$. For the preparation of calibration curve, 1 ml of each of the different concentration of standard gallic acid solution in ethanol as 100, 50, 25, 10, 5, 2.5 and 1 µg/ml was mixed with 5 ml Folin-Ciocalteu reagent (diluted ten fold) and 4 ml of sodium carbonate solution (7.5 g/ml). The absorbance of the blue colored solution was measured after 30 min at 20ºC at 765 nm in Schimadzu 1700 UV-Visible spectrophotometer and the calibration curve was constructed between concentration versus absorbance.

The same procedure was applied for both types of test formulations of Draksharishta as Draksharishta-T and Draksharishta-M respectively and for its marketed preparation, 1 ml of each of the test preparation of Draksharishta as Draksharishta-T (1g/100ml), Draksharishta-M (1g/100ml) and its marketed preparation (1g/100 ml) was mixed with the same reagents as did in the construction of calibration curve and after 1 h, the absorbance of blue colored solution formed was measured for the determination of total phenolic content in both types of Draksharishta as Draksharishta-T, Draksharishta-M and in its marketed preparation. All determinations were performed in triplicate. The total phenolic content was determined in terms of
gallic acid equivalent (GAE) as milligram per gram of the test formulation and expressed as percentage weight by weight (%w/w).

**Estimation of total flavonoid content**

Total flavonoid content was estimated in both types of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively and in its marketed preparation by aluminium chloride method\(^{16}\). For the preparation of calibration curve, 1 ml of each of the different concentration of standard quercetin solution in methanol as 10, 20, 40, 60, 80 and 100 µg/ml was added to the 10 ml capacity volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3 ml of 5% sodium nitrite (NaNO\(_2\)) was added. After 5 min, 0.3 ml of 10% aluminium chloride (AlCl\(_3\)) was added. After 6 min, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against a separately prepared reagent blank at 510 nm.

The same procedure was applied for both of the test formulations of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively and its marketed preparation, 1 ml of each of the test preparation of Draksharishta as Draksharishta-T (1g/10ml), Draksharishta-M (1g/10ml) and marketed Draksharishta (1g/10ml) was mixed with the same reagents as did in the construction of calibration curve, and then the absorbance was measured for the determination of total flavonoid content in both the test formulations of Draksharishta and in its marketed preparation. All determinations were performed in triplicate. The total flavonoid content was measured in terms of quercetin equivalent as milligram per gram of the test formulation and expressed as percentage weight by weight (%w/w).

**In vitro determination of antioxidant activity**

**Super-oxide radical scavenging activity**

The super-oxide radical scavenging activity of both types of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively and marketed Draksharishta was measured by NBT method\(^{17}\). About 1 ml NBT solution containing 156µM NBT dissolved in 1 ml 10mM phosphate buffer, pH 7.4 and 0.1 ml of different concentration as 100, 150, 200, 250 and 300 µg/ml of each of the test preparation of Draksharishta as Draksharishta-T, Draksharishta-M and marketed Draksharishta respectively and standard antioxidant Vitamin-E was mixed and the reaction was started by adding 100 µl of phenazine methosulphate in 100 mM phosphate buffer having pH 7.4. The reaction mixture was incubated at 25°C for 5 min, and absorbance at 560 nm was measured against control sample. Percentage inhibition was determined by comparing the results of test and control as per the formula mentioned below-

\[
\text{Inhibition} \ (%) = \frac{(\text{Control absorbance} - \text{Test absorbance})}{\text{Control absorbance}} \times 100
\]
Assay of lipid per-oxidation

The extent of lipid per-oxidation in goat liver homogenate was measured in vitro in terms of formation of thiobarbituric acid reactive substance (TBARS) by using standard method with the help of spectrophotometer. Goats liver was purchased from local slaughter house. Its lobes were dried between blotting paper and were cut into small pieces with a heavy duty blade. They were then homogenized in glass-teslin homogenizing tubes in cold phosphate buffer saline (pH 7.4). It was centrifuged at 2000 rpm for 10 minutes, and supernatant was diluted with phosphate buffer saline up to final concentration of protein 0.8-1.5mg/0.1ml. Protein concentration was measured by using standard method. To study the comparative response, five different concentrations as 100, 150, 200, 250 and 300 µg/ml of each of the test preparation of Draksharishta as Draksharishta-T, Draksharishta-M prepared by traditional and modern methods respectively and marketed Draksharishta were taken in this experiment. Liver homogenate was aliquoted to seventeen different glass Petri dishes. The first two groups were treated as control and standard where buffer and Vitamin-E were added respectively. From the 3rd group upto 7th group, different concentration as (100, 150, 200, 250 and 300 µg/ml) of Draksharishta-T, from 8th group upto 12th group different concentration as (100, 150, 200, 250 and 300 µg/ml) of Draksharishta-M while from 13th to 17th group various concentration as (100, 150, 200, 250 and 300 µg/ml) of marketed Draksharishta were added.

Lipid per-oxidation was initiated by adding 100 µl of 15 mM ferrous sulphate solution to 3 ml of liver homogenate. After 30 minutes, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10% trichloro acetic acid. After 10 minutes, tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% thiobarbituric acid. The mixture was heated in a water bath to complete the reaction. The intensity of pink colored complex formed was measured at 535 nm. The percentage of inhibition of lipid per-oxidation was calculated by the following formula –

\[
\text{Inhibition (\%)} = \left( \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \right) \times 100
\]

Results

Results of estimation of total phenolics and flavonoids in Draksharishta-T, Draksharishta-M and marketed Draksharishta

Total phenolic content and flavonoids have been estimated in both types of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively and in its marketed preparation. The calibration curve of standard gallic acid for the estimation of total phenolics has been shown in Fig. 1 while calibration curve of standard quercetin for the quantification of total flavonoids has been shown in Fig. 2. A good linear relationship was observed between absorbance and concentration of standard gallic acid in the range of 1 to 100 µg/ml while for quercetin in the range of 10 to 100 µg/ml. Total phenolic content was measured in the terms of gallic acid equivalent (GAE) in milligram per gram of test formulation and was expressed percentage weight by weight (% w/w) while total
flavonoid content was measured in terms of quercetin equivalent in milligram per gram of the test formulation and was expressed as % w/w. Total phenolic content and flavonoids were found present in rich concentration in both types of Draksharishta as Draksharishta-T, Draksharishta-M and in its marketed preparation. Results of total phenolic content and total flavonoids in both types of Draksharishta as Draksharishta-T, M and marketed Draksharishta have been shown in Table 1.

Results of in vitro antioxidant activity of Draksharishta-T, Draksharishta-M and marketed Draksharishta
Both types of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively were evaluated for their antioxidant potential and showed dose dependent antioxidant activity in super oxide radical scavenging activity as well as in lipid per oxidation assay.

Super oxide radical scavenging activity
Effect of both types of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively on super-oxide radical scavenging activity has been shown in Fig.3. Both types of Draksharishta as Draksharishta-T and Draksharishta-M showed significant scavenging of super oxide radical in dose dependent manner and showed inhibitory concentration (IC$_{50}$) 138.06 µg/ml and 145.35 µg/ml respectively. Marketed Draksharishta also showed significant scavenging of super oxide radical in dose dependent manner and showed IC$_{50}$ 141.75 µg/ml.

Lipid per oxidation assay
Effect of both types of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively on lipid per oxidation assay has been shown in Fig.4. Both types of Draksharishta as Draksharishta-T and Draksharishta-M inhibited the ferrous sulphate induced lipid per oxidation in dose dependent manner and showed inhibitory concentration (IC$_{50}$) 230.03 µg/ml and 236.11 µg/ml respectively. Marketed Draksharishta also significantly inhibited the ferrous sulphate induced lipid per oxidation in dose dependent manner and showed IC$_{50}$ 233.49 µg/ml.
Calibration curve of gallic acid for the estimation of total phenolics

\[ y = 0.0106x + 0.041 \]
\[ R^2 = 0.996 \]

Fig. 1 Calibration curve of standard gallic acid for the estimation of total phenolics

Calibration curve of quercetin for estimation of total flavonoid content

\[ y = 0.0058x + 0.1266 \]
\[ R^2 = 0.9997 \]

Fig. 2 Calibration curve of standard quercetin for the estimation of total flavonoids
**Fig. 3** Effect of Draksharishta-T, M and its marketed formulation on super oxide radical scavenging activity
All values are shown as mean ± SEM of three replicates

**Fig. 4** Effect of Draksharishta-T, M and its marketed formulation on lipid per oxidation model
All values are shown as mean ± SEM of three replicates
Table 1. Total phenolic content and flavonoids in Draksharishta-T, M and marketed Draksharishta

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenolic content (%w/w)</th>
<th>Total flavonoids (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Draksharishta-T</td>
<td>0.0967</td>
<td>0.01163</td>
</tr>
<tr>
<td>Draksharishta-M</td>
<td>0.0961</td>
<td>0.01129</td>
</tr>
<tr>
<td>Marketed Draksharishta</td>
<td>0.0963</td>
<td>0.01112</td>
</tr>
</tbody>
</table>

Discussion

Plants contain a large variety of phyto-nutrients, many having antioxidant properties. Antioxidant compounds include vitamins, carotenoids, flavonoids and phenolics. Among them, phenolics and flavonoids are the most important and exhibit substantial antioxidant activity\textsuperscript{20,21}. Thus, on the basis of presence of rich concentration of total phenolics and flavonoids, both of the test formulations of Draksharishta as Draksharishta-T and Draksharishta-M prepared by traditional and modern methods respectively were evaluated for their antioxidant potential on two different \textit{in vitro} models as super-oxide radical scavenging activity and lipid per-oxidation assay.

\textit{In vitro} superoxide radical scavenging activity

Superoxide radical is a highly toxic species and is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess super oxide dismutase enzymes that catalyze the breakdown of super oxide radical\textsuperscript{22}.

Reduced phenazine methosulphate assay was used to measure the super oxide dismutase activity of Draksharishta-T, M and its marketed preparation and all these test preparations of Draksharishta exhibited dose dependent antioxidant activity.

\textit{In vitro} assay of lipid per oxidation

Lipids are widely involved in oxidative reactions and these reactions can be induced by some radicals, called reactive oxygen species (ROS). Oxidative stress caused by ROS in the living cell is associated with numerous diseases as coronary heart disease, atherosclerosis, inflammation, cancer, anaemia and age related muscular degeneration and ageing. Use of antioxidants can reduced the problems caused by reactive oxygen species and thus they retard the oxidative process\textsuperscript{23}.

The results presented in Fig.4, showed that both types of Draksharishta as Draksharishta-T, Draksharishta-M and its marketed preparation inhibited the ferrous sulphate induced lipid per-oxidation in a dose dependent manner. The inhibition could be caused by the absence of ferryl-perferryl complex or by changing the ratio of ferric to ferrous or by reducing the rate of conversion of ferrous to ferric or by changing the iron itself or combination thereof\textsuperscript{24}. Thus, Draksharishta-T, M and its marketed preparation showed potent antioxidant activity and evidenced that the free radical scavenging potential helps in ameliorating disease process. Therefore, both types of Draksharishta as Draksharishta-T, Draksharishta-M and its marketed preparation can be recommended for the \textit{in vivo} pharmacological activities based on their antioxidant...
potential as cardioprotective activity, hepatoprotective, antidiabetic and many others. Enzyme modifying action of antioxidants could account for their pharmacological activities.

References