

Influence of Age on *Ginkgo Biloba* Phytochemicals in Antimicrobial Activity Perspective

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Abstract

Ginkgo biloba is widely used for its pharmaceutical potential due to its medicinal value. Its medicinal properties are directly related to the presence of the phytochemical constituents, mainly the terpenes trilactone (ginkgolides and bilobalide) and flavonoid glycosides that are likely to be influenced by a number of soil, biogeographic and climatic factors as well as the age of the tree. The present study is aimed to investigate the influence of the age of *Ginkgo* tree with respect to the total phenolic and total flavonoid content, antioxidants and flavonoid glycosides along with the antimicrobial activity following Ultraviolet Spectroscopy and High Performance Liquid Chromatography. The results exhibited great variability in all the phytochemicals, under study, showing significant decline with ageing of the *Ginkgo* tree. However, the antimicrobial activity that was determined in terms of strong inhibition on a range of microorganisms (bacteria, actinomycetes and fungi) in plate assays, varied in this respect to limited extent.

Keywords- *Ginkgo biloba*, antimicrobial activity, antioxidants, phenolics, flavonoid glycosides

1. Introduction

Medicinal plants are considered rich sources of phenolic compounds, and are known to be responsible for antioxidant activity, antimicrobial activity, and many other health benefits (Singh et al., 2008; van Beek and Montoro, 2009; Sati et al., 2012). Detailed screening for chemical constituents leading to antioxidant and antimicrobial activity of most of the known medicinal plants has, however, need to be carried out with respect to their biogeography. Natural antioxidants from plant sources are gaining more attention as compared to synthetic antioxidants such as, butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone, gallic acid esters due to their adverse effects on health (Barlow, 1990).

Ginkgo biloba L. (common name- Maidenhair), referred as living fossil being the only living member of family Ginkgoaceae, is a dioecious plant, and known to grow in temperate conditions up to 600-2400 m amsl. While the natural habitats of this species are known to be in China, Japan, and Korea, limited number of *Ginkgo* trees have been reported from the hilly areas of Indian Himalayan Region (Singh et al., 2008), maximum being in the state Uttarakhand. Traditionally, various parts of this species have been used on an extensive basis almost all over the world. While the leaf, seed, and bark are generally known for its medicinal

properties, that are useful in treating Alzheimer disease, cardiovascular diseases, cancer, blood disorders, and improving memory, the potential of this species in traditional food has also been well recognized (van Beek and Montoro, 2009; Mahadevan and Park, 2008). *Ginkgo* seeds have been listed as a source of medicine since the early Chinese herbal preparations. The leaves of this “living fossil” (according to Darwin) have been medicinally used since the oldest Chinese medica since 2000 B.C. for its use as a natural PAF (Platelet Activating Factor) antagonist (Braquet et al., 1991). *Ginkgo* leaves have been prescribed as preventive medicine from the time as early as 1509 and is still used in the form of tea. Nowadays, extracts of *Ginkgo* leaves can be purchased in Europe and America in the form of film-coated tablets, oral liquids or injectable solutions (van Beek and Montoro, 2009). Besides having rich antioxidant activity, *Ginkgo* leaf extracts have been shown for strong antimicrobial activity as well (Mazzanti et al., 2000; Sati et al., 2012).

Due to the limited number of *Ginkgo* trees growing scattered in Himalaya and its importance in medicine, the species has received considerable attention by some of the research groups working in IHR. The major research aspects have been related to rhizosphere microbiology (Kumar et al., 2009; Pandey et al., 2009), development of propagation packages (Pandey et al., 2014; Gopichand and Meena, 2015), and phytochemical analysis including extraction and optimization procedures (Kaur et al., 2012; Sati et al., 2013a & b). Numerous factors, such as age of the plant, season, microbial attack, grazing, radiation, competition, and nutritional status, have been confirmed to have a collision on the secondary metabolite profile in higher plants (Harborne and Williams, 2000). Among these, level of plant secondary metabolites is influenced by plant age and important secondary bioactive constituents; such as terpene lactone and flavonoid glycosides with particular reference to *Ginkgo biloba* (Wang et al., 1999; Qian et al., 2002; Yao et al., 2012). The present work, assesses the influence of tree age on the phytochemicals composition with respect to antioxidants and antimicrobials in the leaf extracts of *Ginkgo biloba* growing in temperate conditions of IHR.

2. Material and Methods

2.1 Plant Material Collection

Ginkgo biloba leaf samples of different age (ranged between 14-150 years) were collected during rainy season from six locations in Uttarakhand, Indian Himalaya (Table 1). Leaf samples were dried at room temperature and were further grounded to fine powder, using motor and pestle, and stored at 4 °C for further analysis in air tight zip lock bags.

2.2 Chemicals and Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, gallic acid, ascorbic acid and catechin were procured from Sigma–Aldrich (Steinheim, Germany), Sodium carbonate, 2-(N-morpholino) potassium persulphate, ferric chloride, Folin-Ciocalteu reagent, sodium acetate, potassium acetate, aluminum chloride, acetic acid and K₃Fe(CN)₆ from SRL (Mumbai, India), and 2,2'-azinobis (3-ethylbenzothiazol-6-yl) sulphonic acid (ABTS) from Biogene (USA), methanol

and ethanol from Merck Co., (Darms- tadt, Germany). All the chemicals were of analytical grade.

2.3 Extract Preparation for Total Phenols, Flavonoids and Antioxidant Activity

Dried plant material (5 g) was extracted in 100 ml of methanol and kept for continuous stirring for 24 h at room temperature. Extract was centrifuged at 10,000 rpm for 10 min. Supernatant was evaporated using rotary stirrer under reduced pressure, which was further resuspended in 100 ml of methanolic solvent and stored at 4 °C prior to use, within 2 days.

2.4 Extract Preparation for Flavonoid Glycosides

Extraction was performed with 3 ml of conc HCl and 5 mL of H₂O for 2.5 h. The supernatant was hydrolyzed by the reflux to detect the flavonoid glycosides in the HPLC. The final hydrolyzed filtrate was thereof filtered using Whatman filter paper (no. 42). The filtrate was concentrated using a rotary evaporator to obtain constant mass of extract. Concentrated extracts were dissolved in MeOH and sonicated for 15 min at 40 °C. The prepared samples were filtered through a 0.45 µm filter prior to HPLC analysis and kept in air tight containers at 4 °C, until further analysis.

2.5 Determination of Total Phenolic Content

Total phenolic content in all the extracts was determined by Folin-Ciocalteu's calorimetric method (Singleton et al., 1999). In 0.25 ml of diluted methanolic extract, 2.25 ml distilled water and 0.25 ml Folin- Ciocalteu's reagent was added and allowed to stand for reaction up to 5 min. This mixture was neutralized by 2.50 ml of 7% sodium carbonate (w/v) and kept in dark at room temperature for 90 min. The absorbance of resulting blue color was measured at 765 nm using UV-VIS spectrophotometer (Ultrospec 2100 pro). Quantification was done on the basis of standard curve of Gallic acid prepared in 80% methanol (v/v) and results were articulated in milligrams Gallic acid equivalent (GAE) per gram of a leaf sample on dry weight basis.

2.6 Determination of Total Flavonoids

Flavonoid content in all the extracts was determined by aluminium chloride calorimetric method (Chang et al., 2002). Briefly, 0.50 ml of distilled water and 0.50 ml of 10% (w/v) aluminium chloride was added along with 0.10 ml of 1 M potassium acetate and 2.80 ml of distilled water. This mixture was incubated at room temperature for 30 min. The absorbance of resulting reaction mixture was measured at 415 nm using UV-VIS spectrophotometer (Ultrospec 2100 pro). Quantification of flavonoids was done on the basis of standard curve of quercetin prepared in 80% methanol (v/v) and results were expressed in milligrams quercetin equivalent (QE) per gram of a leaf sample on dry weight basis.

2.7 Antioxidant Activity

Antioxidant activity was measured following Rawat et al. (2011) using three different in vitro assays [i. e., 2, 2-azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical and Ferric reducing antioxidant power (FRAP) assay]. Standard curves for all assays were prepared by ascorbic acid and results expressed in milimole of ascorbic acid equivalent per gram dw.

2.8 HPLC Analysis of Flavonoid Glycosides

120 µl extract of each tree was used in triplicate in high performance liquid chromatography (HPLC) system equipped with L-7100 series pump (Merck Hitachi, Japan) and L-7400 series UV-VIS detector (Merck Hitachi, Japan). Phenolic compounds were separated by using 4.6 x 250 mm i.d., 5 µm, Purosphere; C8 column. The mobile phase used for the study was water, methanol and acetic acid in the ratio of 80: 20: 1 and flow rate was 0.8 mL/min in isocratic mode. The spectra of compounds (total three) were recorded at 280 nm for quercetin, kaempferol and isorhamnetin. The identification of phenolic compounds was done with respect of the retention time of corresponding external standard. UV-VIS spectra of pure standard at different concentrations were used for plotting standard calibration curve. The reproducibility of quantitative analysis was 3.5%. The mean value of content was calculated with \pm SE. The result was expressed as milligram per 100 g dw of leaves.

2.9 Evaluation of Antimicrobial Activity

The test microorganisms were taken from the Microbial Culture Collection established in the Microbiology Lab of the Institute (GBPNIHESD). Information about the microbial cultures is given in Table 2. The antimicrobial activity assays were performed following agar disc diffusion method (Sati et al., 2012). The test organisms were raised as broth cultures in respective media, bacteria and actinomycetes in tryptone yeast extract (TY) and fungi in potato dextrose (PD), in conical flasks, incubating at 25°C for 24 h. The microbial test species included: bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Pseudomonas putida*, *Serratia marcescens*), actinomyces (*Rhodococcus* sp., *Streptomyces* sp., *S. griseoluteus*, *S. griseobrunneus*), and fungi (*Aspergillus niger*, *Fusarium oxysporum*, *Paecilomyces variotii*, *Trichoderma vride*). Antimicrobial activity was performed on Tryptone yeast extract (TY) and Potato dextrose (PD) agar plates through disc diffusion assays at 25 °C for 120 h.

2.10 Statistical Analysis

All the experiments were done in triplicates. Means and standard errors, and one way analysis of variance (ANOVA) were calculated using SPSS 16 software.

3. Results and Discussion

Plants rich in secondary metabolites, including phenolics, flavonoids and carotenoids, have antioxidant activity due to their redox properties and chemical structures. The statistical analysis (Figure 1A&B; Table 2) showed that tree age has a significant influence on total

phenolic, total flavonoids, antioxidants and *Ginkgo* flavonoids glycosides content of the extracts ($p \leq 0.05$). Analyzing the mean of the total samples, a decreasing tendency in production of total phenolic, flavonoid, antioxidants and flavonoids glycosides, with the increasing age, was evident.

Across the *Ginkgo* age series, the higher content of total phenolic (60.20 mg/g dw) and total flavonoid (35.38 mg/g dw) was obtained from the leaf extracts of 14 year old tree (Figure 1A). Similarly, antioxidant activity results revealed that highest antioxidant activity was found in 14 year tree leaf extracts (Figure 1B) (average value ABTS 10.57; DPPH 2.00; FRAP 2.15 mM AAE/ g dw) followed by 35, 45, 85, 120,150 year old trees.

In case of flavonoids glycosides namely, quercetin, kaempferol and isorhamnetin, the extraction was done by reflux method. The maximum recovery of quercetin (181.89 mg/100g dw) and isorhamnetin (167.03 mg/100g dw) was in 14 year tree extract and kaempferol content was higher in 35 year tree extract (194.46 mg/100g dw). Total terpene lactones and flavonol glycosides in different age *Ginkgo* trees have been reported from different countries (Wang et al., 1999; Qian et al., 2002; Yao et al., 2012). The results obtained in the present study are in agreement with the earlier reports in respect to the influence of ageing on *Ginkgo* phytochemicals. In these reports, total terpene lactones and flavonol glycosides content in the leaves of young *Ginkgo* trees were higher than those in the older trees. Nadeem et al. (2002) have reported the influence of age of the tree on taxol content of *Taxus baccata*, with maximum (statistically significant) taxol content in the bark samples collected from trees of >110 yrs. Variation in the active ingredients as in case of *T. baccata* or other phytochemicals, such as total phenolic flavonoids content and the antioxidants, with reference to the age of the plant species is an important factor to be considered while studying the accumulation of secondary metabolites in a particular plant species.

The age and the stage of growth of the plant generally have an impact on phenolic contents (Blum-Silva et al., 2015). The influence of age and plant development on secondary metabolite contents, and the relative proportions of these chemical components, has been demonstrated by several authors for different plant species (Bowers and Stamp, 1993; Hendriks et al., 1997; Doan et al., 2004).

Effect of age on the antimicrobial activity of the *Ginkgo* crude extracts in plate assays performing against three groups of microorganisms, viz. bacteria, actinomycetes and fungi, is presented in Figure 2. The maximum inhibition of microbial growth with reference to Gram positive bacteria, Gram negative bacteria, actinomycetes and fungi was recorded in the leaf extracts prepared from the 14 year old *Ginkgo* trees (5.03, 4.40, 5.22, 1.41 mm, respectively). This variation may be attributed to many factors such as, the effect of climate, soil composition, age and vegetation cycle stage, on the quality, quantity and composition of extracted product,

and the bacterial species/strains (Masotti et al., 2003; Angioni et al., 2006; Sati et al., 2012). Moreover, the plant-microbe interaction will depend on the plant as well as microbial species.

4. Conclusion

It can be concluded that, the age of the tree is one of the important factors in influencing the accumulation of phytochemicals and the antioxidants as well as the associated antimicrobial activity. The study has limitation of the replicates due to the availability of limited trees in the IHR. While the influence of age on phytochemicals of *Ginkgo* was with a clear cut line (decrease with the increasing age), this influence with respect to antimicrobial activity needs further detailed work. The results of this preliminary study give the ideas for planning of the advance research on the subject line. Research on plant based antimicrobials is emerging as an alternate of the microbe based antibiotics that are now well known for their side effects and the development of drug resistance phenomenon (Pandey and Agnihotri, 2015).

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Location	Age (year)	Reference
Kalika (Almora)	150	(Sati et al., 2012)
Chaubatia (Almora)	130	
Snowview (Nainital)	85	
Glenthorn (Nainital)	45	
High Court (Nainital)	33	
GBPIHED (Kosi)	14	

Table 1. Location and age of *Ginkgo biloba* trees

Age (years) of <i>Ginkgo</i> trees	mg/100g dw		
	Quercetin	Kaempferol	Isorhamnetin
150	72.16±0.28 ^d	69.43±0.55 ^f	49.99±1.43 ^f
130	85.48±0.89 ^e	82.47±0.71 ^e	82.86±1.24 ^e
85	96.18±1.45 ^f	101.62±0.84 ^d	85.59±1.10 ^d
45	108.76±0.99 ^c	182.03±0.28 ^c	125.28±1.12 ^c
35	162.28±1.64 ^b	194.46±0.65 ^a	132.83±1.21 ^b
14	181.89±0.80 ^a	191.48±0.49 ^b	167.04±0.87 ^a

Values are mean ± standard error; different letters in a column indicate significant variation (p<0.05); flavonoids expressed as mg /100g dw

Table 2. Flavonoid glycosides in *Ginkgo* with respect to age

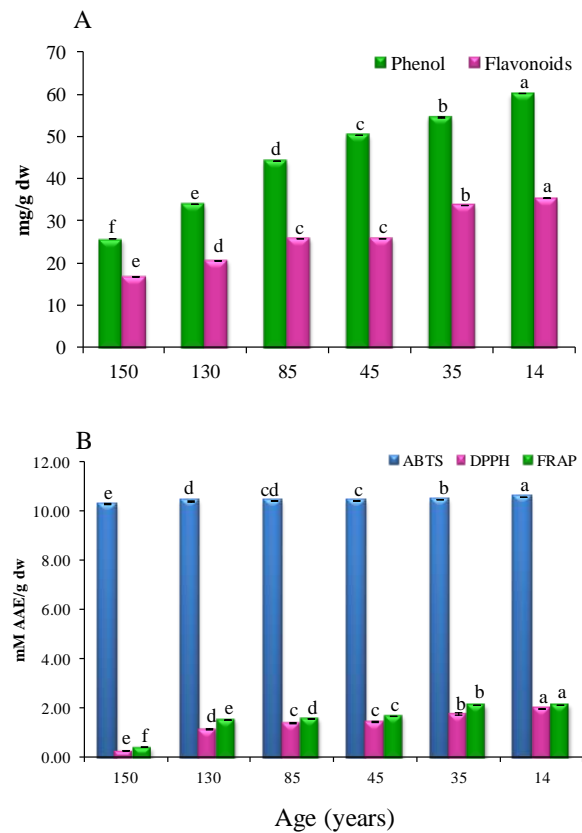


Figure 1. Phytochemicals and antioxidants in *Ginkgo* leaf extracts of different age group trees. A total phenolic and flavonoid contents, and B antioxidant activity (ABTS, DPPH, FRAP); values are mean \pm standard error; different letters in a bar indicate significant variation ($p < 0.05$); total phenolics expressed as mg GAE/g dw; flavonoids expressed as mg QE/g fw, ABTS, DPPH, FRAP activity expressed as mM AAE/ g dw.

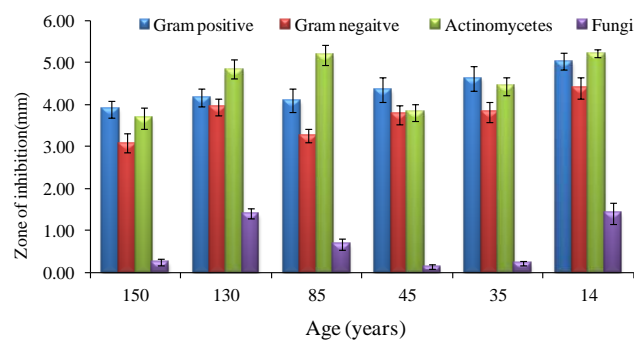


Figure 2. Antimicrobial activity in *Ginkgo* leaf extracts with respect to age; values are mean \pm standard error

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