



Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: <http://www.elsevier.com/locate/jtcm>

Ginkgo biloba flavonoid glycosides in antimicrobial perspective with reference to extraction method

Priyanka Sati, Praveen Dhyani, Indra Dutt Bhatt, Anita Pandey*

Biotechnological Applications, G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora 263 643, Uttarakhand, India

ARTICLE INFO

Article history:

Received 9 May 2017

Received in revised form

26 September 2017

Accepted 2 October 2017

Available online 17 January 2018

Keywords:

Ginkgo biloba

Flavonoid glycosides

Antimicrobials

Antioxidants

HPLC

Indian Himalaya

ABSTRACT

The present study aims to investigate the effect of extraction method on the recovery of flavonoid glycosides, antimicrobials and antioxidants from *Ginkgo* leaves collected from six different locations in Uttarakhand, Indian Himalaya. Four extraction methods, namely maceration, reflux, shaker and soxhlet were considered, where reflux extracts showed higher antimicrobial antioxidant activity and higher content of flavonoid glycosides. The reference standards of *Ginkgo* flavonoid glycosides (quercetin, kaempferol and isorhamnetin) and crude extracts were tested for their antimicrobial activity against gram positive and gram negative bacteria and fungi following disc diffusion method and minimum inhibitory concentration (MIC). All the test microorganisms were observed to be inhibited significantly by *Ginkgo* flavonoids in plate based assays. Correlation coefficients exhibited the extent of contribution of flavonoid glycosides in antimicrobial activity and confirmed the reflux method as a potential method for extraction. Moreover, antioxidant activity as measured by DPPH assay was also found to be higher in reflux method. Significant variation ($p < 0.05$) in the flavonoid glycosides among the locations was also observed and sample collected from GB6 location was found to be the best for quercetin and isorhamnetin, while GB5 for kaempferol. Significant correlation ($r < 0.05$, $r < 0.001$) was obtained while developing the relationship between total flavonoid glycosides and antimicrobials. The present study, thus suggests the reflux method of extraction to be the best for maximum recovery of flavonoid glycosides with higher antioxidant and antimicrobial activities from *Ginkgo* extract.

© 2017 Center for Food and Biomolecules, National Taiwan University. Production and hosting by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Ginkgo biloba L. (common name-maiden hair tree; family-Ginkgoaceae) is a traditionally as well as economically important plant that is now cultivated in China, Japan, Korea, France, Germany and in some parts of India, especially in Uttarakhand state, for its aesthetic and the medicinal value.¹ The medicinal parts of *Ginkgo* (fresh or dried leaves, and seeds separated from their fleshy outer layer) are known for antioxidant, antiasthmatic, wound healing, neuroprotective and antimicrobial properties and to improve the mental capacity in Alzheimer's patients.^{2–4} The medicinal and the antimicrobial properties of *Ginkgo* can be attributed to two

important chemical constituents, viz. terpenes trilactone (ginkgolides and bilobalide) and flavonoid glycosides.⁵ Among other constituents, the flavonoids have received attention in medical research due to the various useful properties, including antiallergic, anti-inflammatory, antioxidants, antimicrobial and oestrogenic activities, enzyme inhibition, and vascular and cytotoxic anti-tumour activities.⁶

The flavonoids may occur in plants in the form of glycosides in several glycosidic combinations. However, an increase in the ratio of aglycones to glycosides in extracts is indicative of degradation. For this reason, hydrolysis has been used to release the aglycones in extracts that can be further investigated by HPLC.⁷ *Ginkgo* flavanoid glycosides have been reported in the forms of mono-, di- and tri-sugar units of quercetin (Q), kaempferol (K) and isorhamnetin (I).⁸

Extraction of bioactive compounds, imparting antimicrobial activity, facilitates the pharmacology studies leading to the synthesis of more potent drugs with reduced toxicity.⁹ The term 'antimicrobial' refers to the inhibition of growth with respect to

* Corresponding author.

E-mail address: anita@gbpihed.nic.in (A. Pandey).

Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

specific groups of microorganisms such as antibacterial, antifungal, antiviral and antiprotozoan. Most of the research related to *Ginkgo* leaf extracts involves either isolation of phytoconstituents or evaluation of pharmacological activities. However, very few studies regarding the correlation of biological activity with the isolated compounds are on record.¹⁰ Although, widespread research is in progress on the utilization of *Ginkgo* as an effective antioxidant, literature on the influence of different extraction methods on the flavonoid glycosides recovery and their antimicrobial and antioxidant activities is negligible. Therefore, the present study investigates the effect of different extraction methods on the antimicrobial, antioxidant, and the extraction yield in *Ginkgo*. The results obtained would help in determining the variation in flavonoids content, antioxidant and antimicrobial activities and selection of suitable extraction method for harnessing the potential of species in pharmaceutical purposes.

2. Material and methods

2.1. Plant material

Ginkgo biloba leaves were collected during rainy season from six locations in Uttarakhand, Indian Himalaya across an altitudinal gradient of (1200–2002 m amsl). The locations are referred as: GB1 (Kalika, Almora), GB2 (Chaubatia, Almora), GB3 (Snowview, Nainital), GB4 (Highcourt, Nainital), GB5 (Glenthorn, Nainital) and GB6 (GBPIHED, Almora). 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

Standard reference compounds of flavonoid (quercetin, kaempferol, isorhamnetin), p-iodo nitro tetrazolium were obtained from Sigma-Aldrich. Ethanol (EtOH), Orthophosphoric acid (H₃PO₄) and Hydrochloric acid (HCl) of analytical grade and Methanol of HPLC grade were obtained from Merck (Mumbai, India). All the extracts and solvents were filtered through 0.45 µm membrane filter (Millipore). Growth media, Tryptone Yeast extract (TY) and Potato Dextrose (PD) was purchased from Hi media, India.

2.3. Extraction procedure

Four extraction methods (maceration, reflux, shaker, and soxhlet) were employed to obtain their respective extracts. In reflux method, extraction was performed with 3 ml of conc HCl¹¹ and 5 mL of H₂O for 2.5 h. Soxhlet extraction was performed in the soxhlet apparatus for 6 h at 60 °C. Shaker extraction was performed at 200 rpm at 25 °C. The liquid extract obtained was then filtered through Whatman filter paper (no. 42). The extractions were carried out with the same solid to solvent ratio of 5 g of the ground leaf sample to 50 mL EtOH (99.7%, v/v). For maceration extraction, the extract was macerated overnight at room temperature. The supernatant of maceration, shaker and soxhlet 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 respective macerated, reflux, soxhlet, and shaker 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.

2.4. HPLC analysis

2.4.1. Standard solutions

Stock solutions 1 mg/ml of quercetin, kaempferol, and isorhamnetin were prepared in methanol, and diluted to obtain the desired working concentrations in the quantification range. The calibration graphs were plotted after linear regression of the peak areas vs concentrations. All the reference standard solutions were stored at –20 °C.

2.4.2. HPLC analysis and chromatographic conditions

HPLC analysis was performed with Chromatographic system (Merck-Hitachi, Japan) consisting of manual sampler with 20 µL injector volume and an UV-VIS detector. The separation was performed on C18 column at ambient temperature. The elution was performed by using isocratic mode with mobile phase (0.05% H₃PO₄/MeOH 50:50)¹¹ and a flow rate of 0.8 mL/min at 32 °C. The samples were run for 40 min at wavelength of 360 nm. The identification of flavonoid glycoside compounds was done with respect to retention time of corresponding external standards. The mean value of content was calculated with ± standard error. The results are expressed as mg/100 g dry weight.

2.5. Determination of antimicrobial and antioxidant activity

2.5.1. Test microorganisms

The test microorganisms were taken from the Microbial Culture Collection established in the Microbiology Lab of the Institute. The microbial cultures were represented by gram negative and gram positive bacteria and fungi. The bacterial cultures were maintained on TY agar slants at 4 °C and also in glycerol stocks at –20 °C, while the fungal cultures were maintained on PD agar slants at 4 °C. The extracts and standard flavonoid glycosides were individually tested against different bacteria and fungi including *Bacillus subtilis* (NRRL B-30408), *Micrococcus roseus* (MTCC8133), *Pseudomonas putida* (MTCC6842), *Serratia marcescens* (MTCC4822), *Fusarium oxysporum* (ITCC4219) and *Trametes hirsuta* (MTCC11397). The test microorganisms were obtained from the Microbial culture collection that has been established in the Microbiology Lab of the Institute (GBPNIHESD). These cultures have also been accessioned in National/International depositories. The accession details are: *Bacillus subtilis* (NRRL B-30408) with Agricultural Research Service (ARS) patent culture collection, United States Department of agriculture, Illinois; *Micrococcus roseus* (MTCC8133), *Pseudomonas putida* (MTCC6842), *Serratia marcescens* (MTCC4822) and *Trametes hirsuta* (MTCC11397) with Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India and *Fusarium oxysporum* (ITCC4219) with Indian Type Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India.

2.5.2. Disc diffusion assay

The antimicrobial activity assays were performed following agar disc diffusion method. The test organisms i.e. bacteria were inoculated in TY and fungi in PD broth in conical flasks, and incubated at 25 °C for 24 h. Simultaneously, TY and PD agar plates were prepared for performing the assays. For testing the antimicrobial activity against the test microorganisms, 25 µl of microbial suspension was inoculated over the agar, spreading uniformly using a glass spreader. Five mm filter paper discs (Whatman paper no. 42), carrying 15 µl suspension of leaf extract were placed on agar surface inoculated with the individual microorganisms. The plates were incubated at 25 °C for 120 h; observations were recorded by measuring the zone of inhibition against the test microorganisms.

2.5.3. Minimum inhibitory concentration (MIC)

The MIC of *Ginkgo* leaf extracts was determined following Clinical and Laboratory Standard Institute methodology.^{12,13} Dilutions, ranging from 9 to 0.100 mg/ml of leaf extracts were prepared in tubes, including one growth control (medium + test organism), and one sterility control (medium + test extracts). The final concentration of microbial culture adjusted to 1.5×10^5 CFU/mL (optical density 0.1 at 600 nm), was then inoculated in different tubes (9–0.100 mg/ml) containing leaf extract and incubated at 25 °C. The MIC values were recorded following addition (40 µL) of 0.2 mg/mL p-iodonitrotetrazolium chloride and incubation at 25 °C for 1–4 h, depending upon the microorganism. Viable

microorganisms reduced the yellow dye to pink colour. MIC defined as the lowest concentration that prevented this change and showing absence of growth in the tubes. This was further confirmed by plating on the respective media.

2.5.4. DPPH radical scavenging assay

The scavenging activity in leaf extracts was evaluated using DPPH assay.¹⁴ The results are expressed as mg ascorbic acid/g dry leaves powder. The leaf extracts in the concentration range 9–0.100 mg/ml were prepared. Aliquot (1 mL) of extract solution was combined with 2 mL methanol and then to 0.25 mL of a 1 mM ethanolic solution of DPPH. The mixture vortexed (1 min) and then

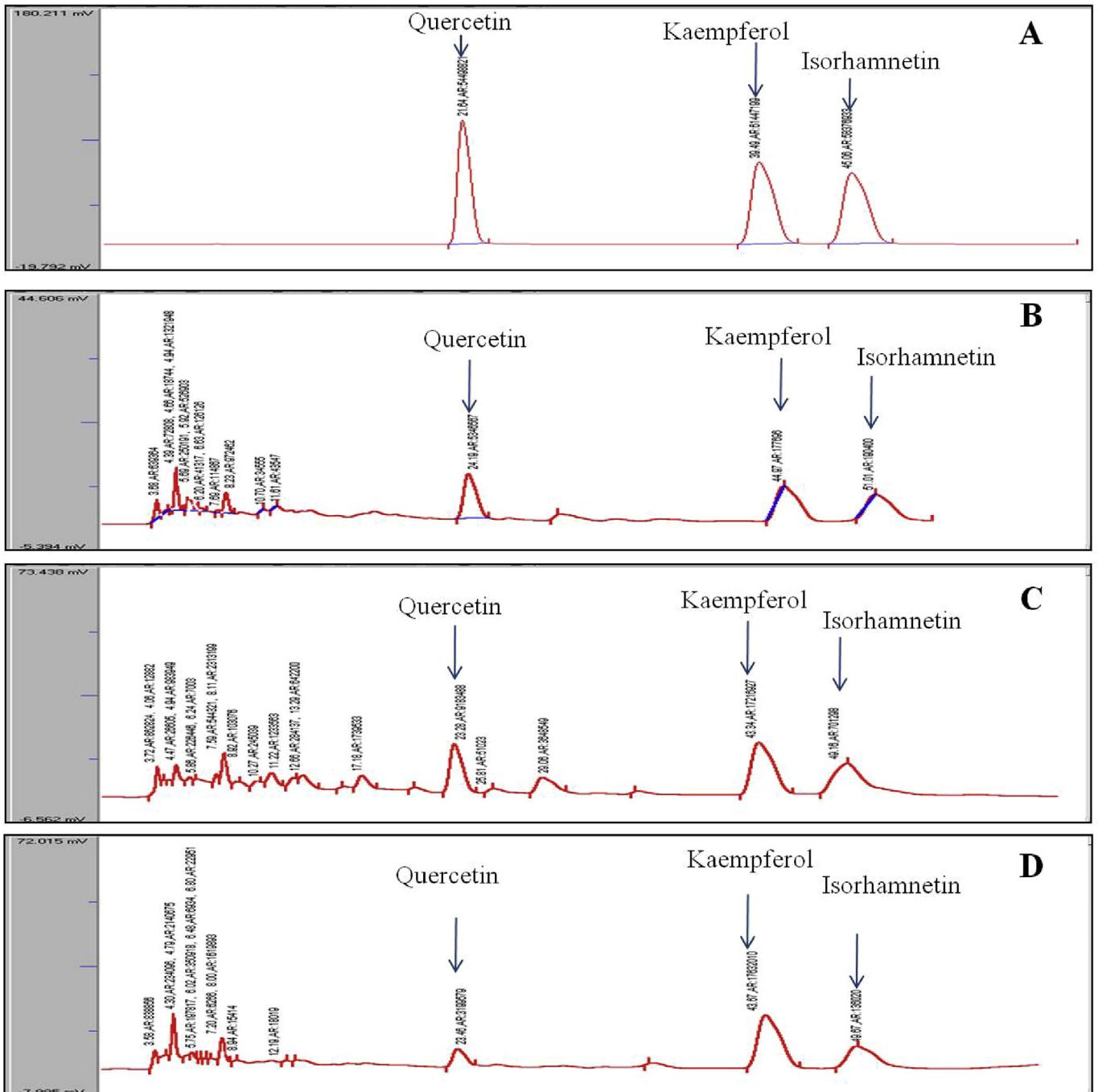


Fig. 1. Representative chromatogram of *Ginkgo* flavonoid glycosides (A) standard compounds (B–D), maceration, reflux and soxhlet extract of *Ginkgo* leaves.

left to stand at room temperature (20 min). Absorbance was measured at 517 nm. Reference sample was prepared with methanol instead of DPPH and the control instead of the extract sample. The free radical scavenging activity of each sample and reference standard was determined as per cent of the inhibition obtained from the following formula:

$$\% \text{ inhibition} = 100 \times (\text{Absorbance}_{\text{blank}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{blank}}$$

IC₅₀ value (mg/mL) is the inhibitory concentration at which DPPH radicals were scavenged by 50 % and was obtained by interpolation from linear regression analysis.

2.6. Statistical analysis

All the experiments were performed in triplicates. Means and standard errors for each sample were calculated. Analysis of variance among means was tested using two way ANOVA (SPSS version 16). Significance level was determined ($p < 0.05$) and significant difference was separated using Duncan's multiple Range Test (DMRT).

3. Results

3.1. Effect of extraction methods on the recovery of flavonoid glycosides

The three flavonoid glycosides in *Ginkgo* leaf extracts varied with respect to the extraction methods (Fig. 1). The reflux extracts showed the highest recovery of quercetin, kaempferol, and isorhamnetin (117.791, 136.915, and 107.265 mg/100 g dw, respectively), while the lowest values were recorded in the macerated extracts (Fig. 2). The total flavonoid glycosides content (quercetin + kaempferol + isorhamnetin) from the six locations varied significantly ($p < 0.05$) with respect to extraction methods. The recovery of total flavonoid glycosides content during all the extraction methods was in an order of reflux > maceration > soxhlet (Table 1). However, in case of the orbital shaker method the concentration of *Ginkgo* flavonoid

Table 1

Variation in concentration of total flavonoid glycosides with different extraction method and location.

Locations	Extraction Methods (mg/100 g dw)		
	Maceration	Reflux	Soxhlet
GB1	7.210 ± 0.216 ^f	71.097 ± 0.847 ^d	8.493 ± 0.538 ^d
GB2	9.961 ± 0.110 ^e	59.942 ± 0.557 ^e	8.566 ± 0.050 ^d
GB3	15.402 ± 0.334 ^d	51.906 ± 0.174 ^f	16.748 ± 0.319 ^c
GB4	24.665 ± 1.068 ^c	107.380 ± 0.164 ^c	29.631 ± 0.144 ^a
GB5	30.699 ± 0.512 ^b	123.640 ± 0.344 ^b	29.605 ± 0.488 ^a
GB6	36.213 ± 1.217 ^a	134.240 ± 0.383 ^a	27.224 ± 0.034 ^b
Total	20.692 ± 2.597	91.367 ± 7.730	20.045 ± 2.241
f- value	268.054	5414	937.766

Values are mean ± standard error; GB1, Kalika; GB2, Chaubatia; GB3, Snowview; GB4, Highcourt; GB5, Glenthorn; GB6, GBPIHED, Kosi; mean values followed by the same letter(s) in a column are not significantly different ($p < 0.05$) based on DMRT.

glycosides was not detectable.

The quantity of different flavonoid glycosides also varied significantly ($p < 0.05$) among the locations with respect to extraction method (Fig. 3A–C). The quercetin and kaempferol content of maceration extracts (Fig. 3A) were higher in GB5 (38.70 and 68.47 mg/100 g dw) while isorhamnetin was higher in GB6 (46.68 mg/100 g dw), minimum content was estimated in GB1 location (quercetin (9.75 mg/100 g dw), kaempferol (6.10 mg/100 g dw), and isorhamnetin (11.98 mg/100 g dw)). In case of reflux extracts (Fig. 3B) the quercetin (181.89 mg/100 g dw) and isorhamnetin (167.03 mg/100 g dw) was higher in GB6 and kaempferol content was higher in GB5 (194.46 mg/100 g dw) location. Minimum content of quercetin (72.16 mg/100 g dw) and isorhamnetin (50.00 mg/100 g dw) was estimated in GB3, while kaempferol (69.43 mg/100 g dw) was minimum in GB2 location. In case of soxhlet extraction (Fig. 3C), the quercetin content (15.55 mg/100 g dw) was higher in GB6 and lowest in GB2 location (2.96 mg/100 g dw). The kaempferol was higher in GB4 (61.89 mg/100 g dw) and GB1 (63.32 mg/100 g dw) locations and isorhamnetin in GB4 (38.58 mg/100 g dw). The lowest kaempferol content was estimated in GB1 (22.12 mg/100 g dw) and GB2 (22.56 mg/100 g dw) and isorhamnetin in GB2 (6.74 mg/100 g dw) location.

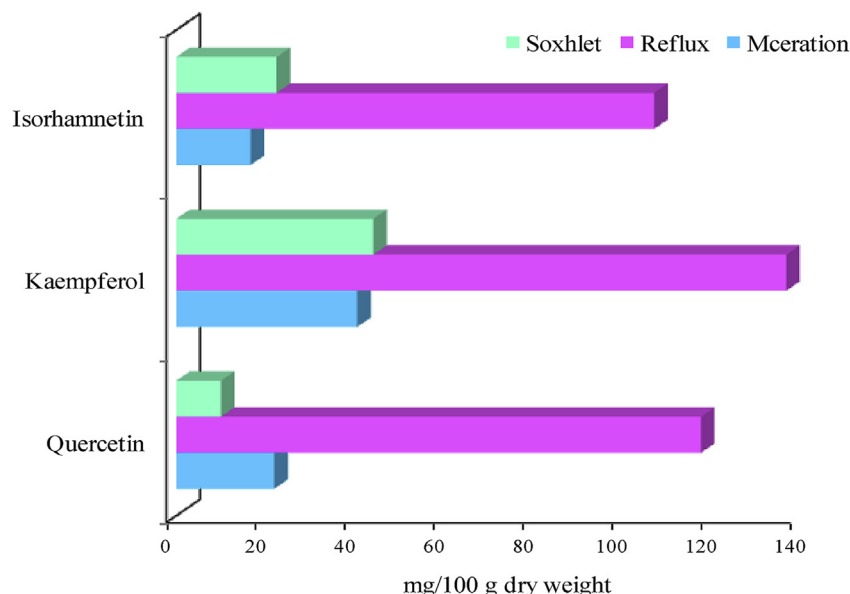


Fig. 2. Flavonoid glycosides (Quercetin Kaempferol and Isorhamnetin) obtained using different extraction methods.

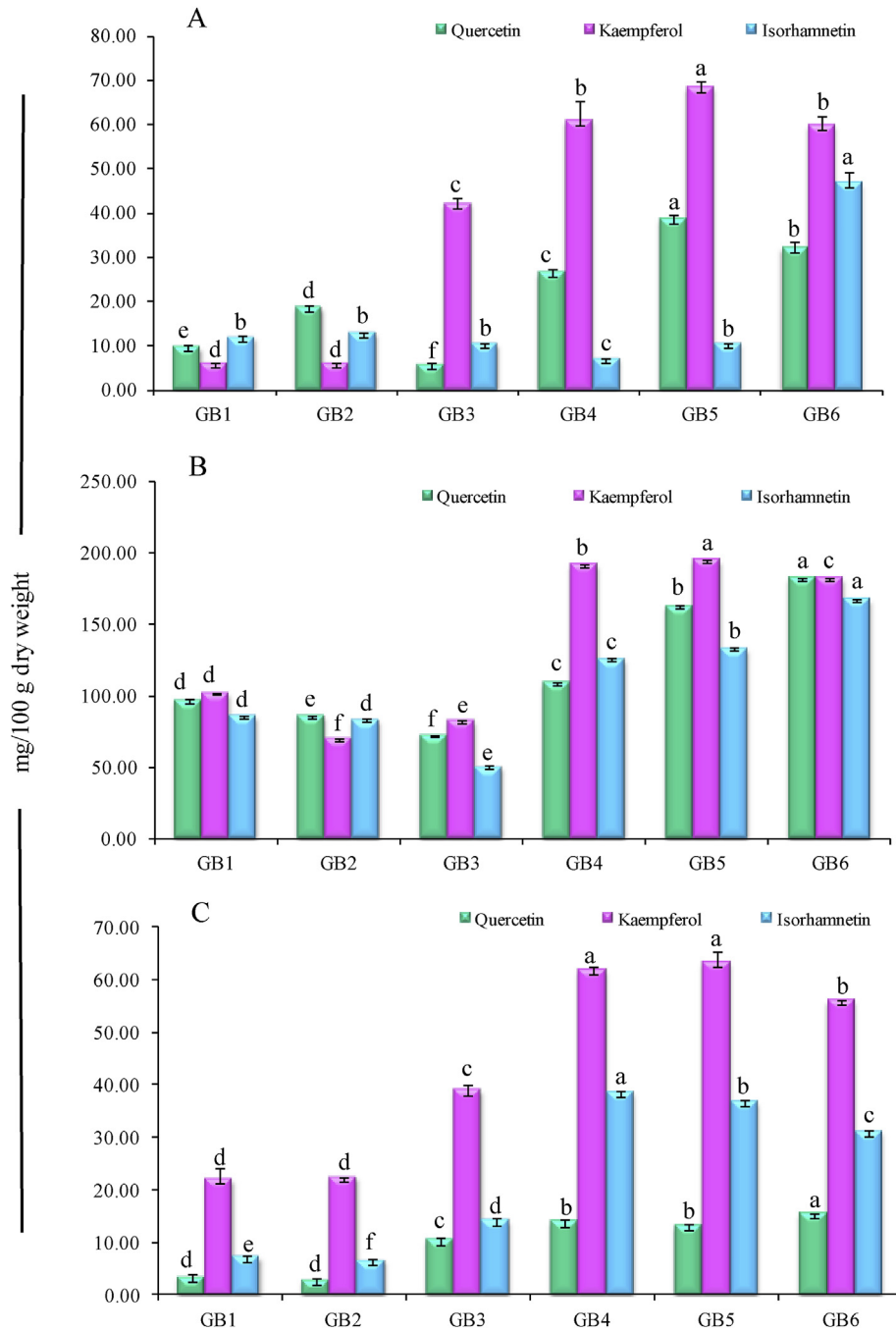


Fig. 3. *Ginkgo* flavonoid glycosides with different locations (a) Maceration (b) Reflux and (c) Soxhlet, values are mean ± standard error; mean values followed by the same latter(s) in a column are not significantly different ($p < 0.05$) based on DMRT.

3.2. Effect of extraction methods on antimicrobial and antioxidant properties of *Ginkgo* flavonoid glycosides

Regarding the antimicrobial activity of 3 major *Ginkgo* flavonoid glycosides (quercetin, kaempferol, isorhamnetin and mixture of all three), the zone of inhibition for mixture ranged between 2-5 mm, while for isorhamnetin, quercetin and kaempferol it was recorded between 1.0-2.47 mm, 1.0–2.67 mm and 1.0–1.93 mm, respectively (Table 2a and Fig. 4), hence, the mixture showing highest antimicrobial activity (synergistic) for all the test microorganisms. Quercetin and isorhamnetin exhibited the highest zone of inhibition against *B. subtilis*, while *F. oxysporum* was inhibited maximum by

Table 2a

Disc diffusion assay for the determination of antimicrobial activity of standard compounds and their mixture.

Microorganisms	Zone of inhibition (mm)			
	Quercetin	Kaempferol	Isorhamnetin	Mixture
<i>Bacillus subtilis</i>	2.67 ± 0.33	1.33 ± 0.58	2.47 ± 0.33	5.00 ± 0.00
<i>Micrococcus roseus</i>	2.33 ± 0.33	1.00 ± 0.33	2.00 ± 0.33	4.00 ± 0.57
<i>Pseudomonas putida</i>	1.67 ± 0.33	1.37 ± 0.33	2.33 ± 0.33	5.00 ± 0.00
<i>Serratia marcescens</i>	2.00 ± 0.00	1.93 ± 0.33	2.00 ± 0.33	4.33 ± 0.00
<i>Fusarium oxysporum</i>	2.33 ± 0.33	1.67 ± 0.33	1.00 ± 0.00	3.00 ± 0.00
<i>Trametes hirsuta</i>	1.00 ± 0.00	1.33 ± 0.33	1.00 ± 0.00	2.00 ± 0.00

Values are mean ± standard error (n = 3).

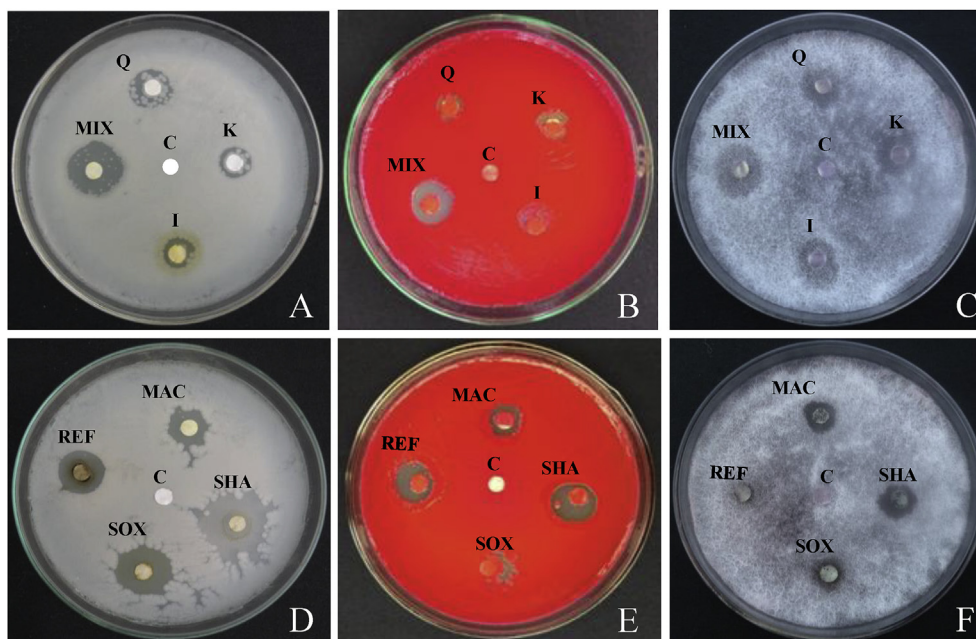


Fig. 4. (A–C) Antimicrobial activity in *Ginkgo* standards- Quercetin (Q), Kaempferol (K), Isorhamnetin (I), Mixture of all the three standards (MIX) and (D–F) leaf extracts with different extraction methods (Maceration: MAC, Reflux: REF, Shaker: SHA and Soxhlet: SOX) and organic solvent (c) against: *Bacillus subtilis*, *Serratia marcescens* and *Fusarium oxysporum*, respectively.

quercetin and kaempferol. Kaempferol showed maximum activity against *P. putida*, while isorhamnetin had highest zone of inhibition for *M. roseus* and *S. marcescens*. The mixture had highest zone of inhibition for *B. subtilis* and *P. putida*.

Antimicrobial activity in *Ginkgo* leaf extracts was also found to be affected by the type of extraction method (Table 2b and Fig. 4). Among the 4 methods used, the reflux crude extracts from all different locations exhibited highest inhibition against the test microorganisms followed by maceration, soxhelt and shaker methods, respectively, with reflux extracts showing inhibition zone ranging from 1.67 ± 0.33 to 7.33 ± 0.33 mm. The crude extracts had highest antimicrobial activity against the Gram + ve bacteria (*B. subtilis*) followed by Gram –ve bacteria (*P. putida*) and fungi. Further, correlation analysis (Table 3) revealed that zone of inhibition of standards mixture and all plant crude extracts activity was

positively correlated with each other's ($p < 0.01$). Isorhamnetin inhibition activity was also correlated with the *Ginkgo* plant crude extracts activity ($p < 0.01$, $p < 0.05$). In MIC experiments, the reflux extracts showed the lowest inhibitory concentration against all the test microorganisms with the lowest value (0.183 mg/mL) in case of *B. subtilis* and the highest (0.300 mg/mL) in case of *F. oxysporum* (Table 4). As displayed in Table 5, the value for the antioxidant activity showed wide variability ranging between 0.325 mg/mL (reflux extract) and 6.5 mg/mL (shaker) by DPPH assay.

3.3. Factorial analysis and relationship between total flavonoid glycosides and antimicrobial activity against test microorganisms

Factorial analysis revealed that the location and the method of extraction, individually and in interaction, significantly ($P < 0.05$)

Table 2b

Disc diffusion assay for the determination of antimicrobial activity of *Ginkgo* plants.

Microorganisms	Zone of inhibition (mm)											
	GB1				GB2				GB3			
	Mac	Ref	Sha	Sox	Mac	Ref	Sha	Sox	Mac	Ref	Sha	Sox
<i>B. subtilis</i>	4.33 ± 0.67	6.67 ± 0.58	4.33 ± 0.33	4.33 ± 0.33	4.00 ± 0.33	6.67 ± 0.33	4.27 ± 0.00	5.33 ± 0.33	5.33 ± 0.33	6.33 ± 0.33	4.00 ± 0.00	5.33 ± 0.33
<i>M. roseus</i>	3.00 ± 0.00	5.67 ± 0.33	4.00 ± 0.33	4.00 ± 0.00	2.33 ± 0.33	5.33 ± 0.33	3.67 ± 0.58	4.33 ± 0.58	3.67 ± 0.33	6.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.58
<i>P. putida</i>	2.67 ± 0.33	5.00 ± 0.58	4.67 ± 0.00	4.00 ± 0.33	3.67 ± 0.33	4.67 ± 0.67	4.33 ± 0.33	2.33 ± 0.67	1.67 ± 0.33	4.67 ± 0.67	3.00 ± 0.00	4.67 ± 0.33
<i>S. marcescens</i>	2.00 ± 0.00	4.00 ± 0.58	3.67 ± 0.33	3.00 ± 0.33	2.67 ± 0.33	5.67 ± 0.33	3.00 ± 1.00	4.00 ± 0.33	2.00 ± 0.00	4.33 ± 0.67	2.67 ± 0.33	3.67 ± 0.33
<i>F. oxysporum</i>	0.00	1.67 ± 0.33	0.67 ± 0.33	1.67 ± 0.33	2.00 ± 0.00	2.00 ± 0.00	1.67 ± 0.58	1.33 ± 0.33	2.00 ± 0.33	2.67 ± 0.00	1.67 ± 0.33	0.67 ± 0.33
<i>T. hirsuta</i>	1.67 ± 0.33	2.33 ± 0.33	1.00 ± 0.00	1.33 ± 0.58	2.00 ± 0.00	3.00 ± 0.33	2.67 ± 1.00	1.00 ± 0.58	0.00	3.00 ± 0.00	1.67 ± 0.33	1.00 ± 0.00
Microorganisms	GB4				GB5				GB6			
	Mac	Ref	Sha	Sox	Mac	Ref	Sha	Sox	Mac	Ref	Sha	Sox
	<i>B. subtilis</i>	5.00 ± 0.00	7.33 ± 0.00	4.57 ± 0.00	5.67 ± 0.00	5.67 ± 0.33	6.67 ± 0.33	4.00 ± 0.00	5.00 ± 0.00	3.33 ± 0.33	7.00 ± 0.33	4.00 ± 0.00
<i>M. roseus</i>	3.33 ± 0.33	5.00 ± 0.58	4.33 ± 0.33	4.57 ± 0.33	3.90 ± 0.58	6.33 ± 0.33	4.00 ± 0.58	5.00 ± 0.00	3.33 ± 0.33	5.33 ± 0.67	4.67 ± 0.33	4.67 ± 0.33
<i>P. putida</i>	4.00 ± 0.00	4.67 ± 0.33	4.00 ± 0.58	4.00 ± 0.33	3.75 ± 0.33	4.67 ± 0.33	2.67 ± 0.00	3.67 ± 0.33	3.67 ± 0.33	5.00 ± 0.00	4.00 ± 0.00	3.67 ± 0.33
<i>S. marcescens</i>	3.00 ± 0.00	4.33 ± 0.33	4.33 ± 0.33	3.00 ± 0.67	3.33 ± 0.67	5.00 ± 0.00	3.33 ± 0.33	3.67 ± 0.33	2.67 ± 0.33	4.33 ± 0.33	3.67 ± 0.33	4.00 ± 0.00
<i>F. oxysporum</i>	2.00 ± 0.58	2.67 ± 0.33	2.67 ± 0.67	2.00 ± 0.00	2.67 ± 0.58	3.67 ± 0.58	2.00 ± 0.33	2.00 ± 0.00	0.00	2.33 ± 0.00	0.00	2.33 ± 0.33
<i>T. hirsuta</i>	2.33 ± 0.33	3.33 ± 0.33	1.15 ± 0.33	3.33 ± 0.33	3.00 ± 0.33	2.33 ± 0.67	1.67 ± 0.00	2.67 ± 0.33	2.33 ± 0.67	3.67 ± 0.58	2.00 ± 0.00	2.33 ± 0.33

Values are mean ± standard error (n = 3); GB1, Kalika; GB2, Chaubatia; GB3, Snowview; GB4, Highcourt; GB5; Glenthorn; GB6, GBPIHED, Kosi; Mac, Maceration; Ref, Reflux; Sha, Shaker; Sox, Soxhlet.

Table 3

Correlation coefficients among Quercetin, Kaempferol, Isorhamnetin, mixture of all three standards and *Ginkgo* plants reflux extracts with respect to different microorganisms antimicrobial activity.

	Antimicrobial activity of different microorganisms									
	Standards				Crude reflux extracts					
	Quercetin	Kaempferol	Isorhamnetin	Mixture	GB1	GB2	GB3	GB4	GB5	GB6
Quercetin	1									
Kaempferol	0.234	1								
Isorhamnetin	0.339	0.483*	1							
Mixture	0.577*	0.451	0.609**	1						
GB1	0.356	0.3290	0.702**	0.831**	1					
GB2	0.335	0.359	0.780**	0.691**	0.904**	1				
GB3	0.201	0.146	0.570*	0.744**	0.874**	0.760**	1			
GB4	0.326	0.334	0.776**	0.733**	0.802**	0.802**	0.787**	1		
GB5	0.492*	0.377	0.849**	0.775**	0.860**	0.865**	0.727**	0.807**	1	
GB6	0.472*	0.192	0.755**	0.709**	0.819**	0.796**	0.765**	0.897**	0.862**	1

Level of significance: *- p < 0.05; **-p<0.01.

Table 4

Minimum inhibitory concentration (MIC) of *Ginkgo* different extracts.

Microorganisms	MIC (mg/ml)			
	Maceration	Reflux	Shaker	Soxhlet
<i>Bacillus subtilis</i>	0.300	0.183	0.500	0.500
<i>Micrococcus roseus</i>	0.750	0.216	0.600	0.500
<i>Pseudomonas putida</i>	0.500	0.250	0.500	0.400
<i>Serratia marcescens</i>	0.500	0.283	0.500	0.500
<i>Fusarium oxysporum</i>	0.800	0.300	0.600	0.600
<i>Trametes hirsute</i>	0.850	0.290	0.650	0.700

MIC, minimum inhibitory concentration.

Table 5

Antioxidant activity of the *Ginkgo* leaf extracts determined IC₅₀ DPPH values.

Samples	IC ₅₀ (mg/ml)
Maceration	6.00
Reflux	0.325
Shaker	6.500
Soxhlet	1.200

affects the recovery of quercetin, kaempferol and isorhamnetin from crude extracts with highest f value, ranging from 250.45 to 794.07 (Table 6). The linear regression analysis revealed that total flavonoid glycosides contribute 5.42–50.35% of antimicrobial activity against test microorganisms ($r^2 = 0.503$ for *B. subtilis*, 0.097 for *M. roseus*, 0.333 for *P. putida*, 0.257 for *S. marcescens*, 0.054 for *F. oxysporum* and 0.256 for *T. hirsute*) (Fig. 5A–F).

4. Discussion

Although flavonoid glycosides have been reported to vary with age, altitude, environmental factors, the extraction yield,

antimicrobial and antioxidant activity has been reported to vary with the method of extraction¹⁵ and selection of solvents. Best bioactivity of *Ginkgo* has been reported from methanol extracts.³ In concurrence, Boonkaew and Camper¹⁶ reported absence of inhibitory activity against *E. coli* in leaf and root tissue methanolic extracts of *Ginkgo*, whereas the extracts from leaf and root derived callus inhibited the growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus* sp., and *Streptococcus pyogenes*. Selection of suitable extraction method has been emphasized with respect to the biological activities.¹⁷ In *Ginkgo*, methods namely simulated digestion process, microwave extraction, soxhlet, maceration ultrasound assisted extraction and orbital shaker have been used for recovery of flavonoids.^{15,18} The present study finds the effect of extraction on the recovery of flavonoids with an influence on antimicrobial and antioxidant activities. The reflux extracts with higher recovery, antimicrobial potential against all the test microorganisms and antioxidant activity can be further attributed to the higher content of flavonoid glycosides in reflux extracts. The maximum recovery from reflux extracts could be due to the maximum hydrolysis of flavonoid glycosides content from the aglycone concentration,¹⁹ requiring shorter time for extraction in comparison to other methods.

In the present study, orbital shaker method was not found suitable for the extraction of *Ginkgo* flavonoid glycosides which might be due to the long extraction time with fewer yields as compared to reflux method. The hot solvent systems under reflux condition are likely to be more efficient for the recovery of *Ginkgo* flavonoid glycosides offering higher extraction yield and antimicrobial and antioxidant activities. These observations are in agreement with the earlier findings of Kaur et al.¹⁵ that reported the higher efficiency of reflux with 60% aqueous ethanol in comparison to maceration, ultrasound/orbital shaker and microwave methods with respect to the extraction of flavonoid glycosides from *Ginkgo*. This can be further associated to an effective extraction under

Table 6

Analysis of variance for determination of the effect of location, methods and their interaction on flavonoid glycosides in *Ginkgo* leaf extracts.

Source of variation	DF	Flavonoid glycosides							
		Quercetin		Kaempferol		Isorhamnetin			
		MS	f value	MS	f value	MS	f value		
Location (L)	5	3543.53	1.94***	10480.43	1.63***	4045.21	1.67***		
Extraction methods (E)	2	62846.77	3.44***	53654.78	8.34***	46271.19	1.91***		
L x E	10	1449.80	794.07***	1610.59	250.45***	1302.12	539.26***		
Error	36	1.83		6.43		2.41			

DF, degree of freedom; MS mean of sum; Level of significance *** p < 0.05.

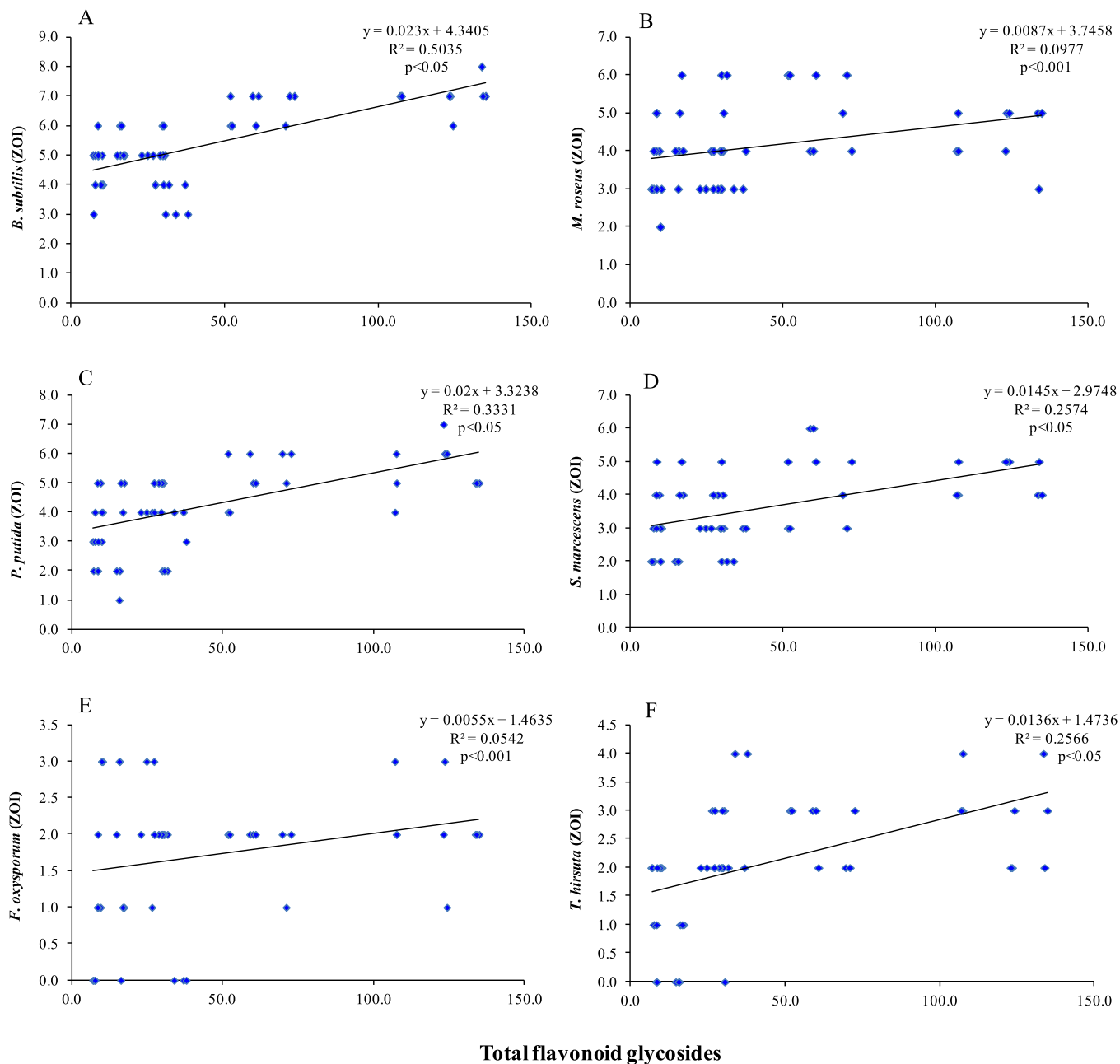


Fig. 5. Relationship between total flavonoid glycosides (mg/g dw) and antimicrobial activity (Zone of inhibition (ZOI = mm) in *Ginkgo* leaf extracts shown as linear correlation between antimicrobial activity and total flavonoid glycosides measured by *B. subtilis* (A), *M. roseus* (B), *P. putida* (C), *S. marcescens* (D), *F. oxysporum* (E), *T. hirsuta* (F).

reflux conditions leading to higher release of some bound phenolics. The individual quercetin, kaempferol and isorhamnetin content in *Ginkgo* leaves, in the present study, have been found on the higher side in different extracts (5.85–68.47 mg/100 g dw in maceration, 69.43–194.46 mg/100 g dw in reflux and 2.96–61.31 mg/100 g dw in soxhlet) in comparison to the earlier report from Poland from green *Ginkgo* leaves.²⁰ In the cited study, the kaempferol, myricetin, and isorhamnetin content predominated in general and ranged from 153 to 661 $\mu\text{g/g}$ d m. extract.

Regarding the antimicrobial activity of *Ginkgo*, the reflux extracts showed higher inhibitory effect on the test microorganisms. The synergistic action of mixture of all the three standards (quercetin, kaempferol and isorhamnetin) was also measured higher in comparison to individual compounds observed in terms of

formation of inhibition zone. The importance of synergistic action of distinct classes of chemical compounds imparting pharmacological activities have been highlighted in many studies. For instance, Tao et al.²¹ reported the antibacterial/antifungal activities and synergistic interaction from *Ginkgo* leaves lipids against *Salmonella enterica*, *Staphylococcus aureus* and *Aspergillus niger*. It is also possible that flavonoid glycosides act in synergy with other polyphenols to boost their antimicrobial activity.²²

Individual compounds have also been screened against variety of microorganisms. In the present study, the individual compounds quercetin, kaempferol and isorhamnetin were found to be active against *B. subtilis*, *M. roseus*, *P. putida*, *S. marcescens*, *F. oxysporum*, *T. hirsuta*; quercetin being more active against all the microorganisms in comparison to isorhamnetin and kaempferol, respectively. Lee

and Kim²³ reported *Ginkgo kaempferol* and *quercetin* to be active against *Clostridium perfringens* and *Escherichia coli*, however, it did not inhibit the anaerobic intestinal and lactic acid bacteria. Choi et al.²⁴ investigated the alkylphenols and ginkgolic acid of *Ginkgo* and showed the antimicrobial activity against *S. aureus*, *Enterococcus faecalis* and *Enterococcus* sp. Fazal et al.²⁵ reported the antibacterial activity of *Ginkgo* leaf extracts against 6 bacteria along with the absence of inhibition in case of *B. subtilis* and *E. coli*. In the present study, all the *Ginkgo* leaf extracts obtained from different methods from different locations exhibited strong inhibition of *B. subtilis*. The sensitivity of gram positive bacteria in comparison to the gram negative to the antimicrobials can be attributed to the lipopolysaccharides found in the outer membrane of the gram-negative bacteria that contribute to the inherent resistance to external agents, such as hydrophilic dyes, antibiotics and detergents.²⁶

In the present study the MIC values ranged from 0.183 to 0.850 mg/ml, lowest being in reflux extract. These results are in line with that of the antimicrobial activity (disc diffusion method) with respect to the three groups of microorganisms. The antioxidant activity of the plant extracts vary according to plant species, geographical and climatic factors.²⁷ The antioxidant properties of *Ginkgo* are attributed to the phenolic compounds and flavonoid glycosides in the extracts.^{15,28} Sati et al.²⁹ have reported the positive correlation between phenolic and flavonoid content and the antioxidant activity. In the present study, antioxidant activity results expressed high flavonoid glycosides in the reflux extracts moderately to highly associate with the antioxidant property.

5. Conclusion

The present study concludes the importance of various procedures in extraction of flavonoid glycosides and determination of antimicrobial and antioxidant activities in *Ginkgo* leaf extracts from different location. The reflux method was the most efficient extraction method for recovery of flavonoid glycosides and also for obtaining the higher antimicrobial and antioxidant activities. The synergistic action of all the 3 individual compounds (*quercetin*, *kaempferol* and *isorhamnetin*) showed higher antimicrobial activity as compared to individual compounds. Further research is needed to identify the mechanism of antimicrobial action of *Ginkgo* flavonoid glycosides.

Conflict of interest

All authors have none to declare.

Acknowledgements

Director, GB Pant National Institute of Himalayan Environment and Sustainable Development, Almora is thanked for extending the facilities. Council of Scientific and Industrial Research (No.: 09/560/(0017)/2013/EMR-I) and Ministry of Environment, Forests and Climate Change, Govt. of India are gratefully acknowledged for financial support.

References

- Singh B, Kaur P, Gopichand Singh RD, Ahuja PS. Biology and chemistry of *Ginkgo biloba*. *Fitoterapia*. 2008;79:401–418.
- Mazzanti G, Mascellino MT, Battinelli L, Coluccia D, Manganaro M, Saso L. Antimicrobial investigation of semipurified fractions of *Ginkgo biloba* leaves. *J Ethnopharmacol*. 2000;71:83–88.
- Sati P, Pandey A, Palmi LMS. Antimicrobial potential of leaf extracts of *Ginkgo biloba* L., growing in Uttarakhand, India. *Natl Acad Sci Lett*. 2012;35:201–206.
- Xu SL, Choi RCY, Zhu KY, et al. Isorhamnetin, a flavonol aglycone from *Ginkgo biloba* L., induces neuronal differentiation of cultured PC12 cells: potentiating the effect of nerve growth factor. *J Evid Based Complement Altern Med*. 2012. <https://doi.org/10.1155/2012/278273>.
- van Beek TA, Montoro P. Chemical analysis and quality control of *Ginkgo biloba* leaves, extracts and phytopharmaceuticals. *J Chromatogr A*. 2009;1216:2002–2032.
- Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents*. 2005;26:343–356.
- Sticher O, Meier P, Hasler A. In: Van Beek TA, ed. *Ginkgo Biloba. The Analysis of Ginkgo Flavonoids*. Amsterdam: Harwood; 2000:179.
- Hasler A, Sticher O. Identification and determination of the flavonoids from *Ginkgo biloba* by high performance liquid chromatography. *J Chromatogr A*. 1992;605:41–48.
- Ebana RUB, Madunagu BE, Ekpe ED, Otung IN. Microbiological exploitation of cardiac glycoside and alkaloids from *Garcinia kola*, *Borreria ocyroides*, *Kola nitida* and *Citrus aurantifolia*. *J Appl Biotechnol*. 1991;71:398–401.
- Bedir ET, Khan RA, Zhao J, et al. Biologically active secondary metabolites from *Ginkgo biloba*. *J Agri Food Chem*. 2002;50:3150–3155.
- Kaur P, Chaudhary A, Singh RD, Prasad GRP, Singh B. Spatial and temporal variation of secondary metabolite profiles in *Ginkgo biloba* leaves. *Chem Biodivers*. 2012a;9:409–417.
- Clinical and laboratory Standards Institute (CLSI). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*. Approved Standard-. Sixth Edition. Wayne: M7-A6; 2005.
- Clinical and laboratory Standards Institute (CLSI). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. Approved Standard-. Third Edition. Wayne: M27-A3; 2008.
- Wong SP, Leong LP, Koh JHW. Antioxidant activities of aqueous extracts of selected plants. *Food Chem*. 2006;99(4):775–783.
- Kaur P, Chaudhary A, Singh B, Gopichand. An efficient microwave assisted extraction of phenolic compounds and antioxidant potential *Ginkgo biloba*. *Natl Product Commun*. 2012b;7:203–206.
- Boonkaew T, Camper ND. Biological activities of *Ginkgo* extracts. *Phytomedicine*. 2005;12:318–323.
- Hayouni EA, Abedrabba M, Bouix M, Hamdi M. The effects of solvents and extraction method on the phenolic contents and biological activities in vitro of Tunisian *Quercus coccifera* L. and *Juniperus phoenicea* L. fruit extracts. *Food Chem*. 2007;105:1126–1134.
- Goh LML, Barlow PJ. Flavonoid recovery and stability from *Ginkgo biloba* subjected to a simulated digestion process. *Food Chem*. 2004;86:195–202.
- Tang D, Yang D, Tang A, et al. Simultaneous chemical fingerprint and quantitative analysis of *Ginkgo biloba* extract by HPLC-DAD. *Anal Bioanal Chem*. 2010;396:3087–3095.
- Kobus J, Flaczyk E, Siger A, Nogala-Kalucka M, Korczak J, Pegg RB. Phenolic compounds and antioxidant activity of extracts of *Ginkgo* leaves. *Eur J Lipid Sci Technol*. 2009;111:1150–1160.
- Tao R, Wang CZ, Kong ZW. Antibacterial/antifungal activity and synergistic interactions between polyphenols and other lipids isolated from *Ginkgo biloba* L. leaves. *Molecules*. 2013;18:216–2182.
- Rauha JP, Remes S, Heinonen M, et al. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int J Food Microbiol*. 2000;56:3–12.
- Lee HS, Kim M. Selective responses of three *Ginkgo biloba* leaf-derived constituents on human intestinal bacteria. *J Agric Food Chem*. 2002;50:1840–1844.
- Choi JG, Jeong SI, Ku CS, et al. Antibacterial activity of hydroxyalkenyl salicylic acids from sarcotesta of *Ginkgo biloba* against vancomycin-resistant *Enterococcus*. *Fitoterapia*. 2009;80:18–20.
- Fazal H, Ahmad N, Ullah I. Antibacterial potential in *Parthenium hysterophorus*, *Stevia rebaudiana* and *Ginkgo biloba*. *Pak J Bot*. 2011;43:1307–1313.
- Negi PS, Jayaprakasha GK, Rao LJM, Sakariah KK. Antibacterial activity of turmeric oil- A byproduct from curcumin manufacture. *J Agric Food Chem*. 1999;47:4297–4300.
- Perry NB, Anderson RE, Brennan NJ, et al. Essential oils from Dalmation sage (*Salvia officinalis* L.): variations among individuals, plant parts, seasons and sites. *J Agric Food Chem*. 1999;47:2048–2054.
- Maltas E, Vural HC, Yildiz S. Antioxidant activity and fatty acid composition of *Ginkgo biloba* from Turkey. *J Food Biochem*. 2011;35:803–818.
- Sati P, Pandey A, Rawat S, Rani A. Phytochemicals and antioxidants in leaf extracts of *Ginkgo biloba* with reference to location, seasonal variation and solvent system. *J Pharm Res*. 2013;7:804–809.