

RESEARCH ARTICLE

Optimized extraction of polyphenolic antioxidants from the leaves of Himalayan Oak species

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OPEN ACCESS

Citation: Pandey A, Belwal T, Tamta S, Rawal RS (2021) Optimized extraction of polyphenolic antioxidants from the leaves of Himalayan Oak species. PLoS ONE 16(11): e0259350. <https://doi.org/10.1371/journal.pone.0259350>

Editor: Christophe Hano, Universite d'Orleans, FRANCE

Received: February 3, 2021

Accepted: October 18, 2021

Published: November 3, 2021

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Data Availability Statement: All relevant data are within the manuscript and its [Supporting Information](#) files.

Funding: The authors received support in the form of facilities from the Director of GBPNiHE. AP and ST received support in the form of funding to initiate the study from IERP cell of GBPNiHE. AP received support in the form of funding from ICIMOD, Nepal through KLCDI-India program.

Competing interests: The authors have declared that no competing interests exist.

Abstract

In this study heat-assisted extraction conditions were optimized to enhance extraction yield of antioxidant polyphenols from leaves of Himalayan *Quercus* species. In initial experiments, a five-factor Plackett-Burman design including 12 experimental runs was tested against the total polyphenolic content (TP). Amongst, X_A : extraction temperature, X_C : solvent concentration and X_E : sample-to-solvent ratio had shown significant influence on yield. These influential factors were further subject to a three-factor-three-level Box-Wilson Central Composite Design; including 20 experimental runs and 3D response surface methodology plots were used to determine optimum conditions [i.e. X_A : (80°C), X_C : (87%), X_E : (1g/40ml)]. This optimized condition was further used in other *Quercus* species of western Himalaya, India. The High-Performance Liquid Chromatography (HPLC) revealed occurrence of 12 polyphenols in six screened *Quercus* species with the highest concentration of catechin followed by gallic acid. Amongst, *Q. franchetii* and *Q. serrata* shared maximum numbers of polyphenolic antioxidants (8 in each). This optimized extraction condition of *Quercus* species can be utilized for precise quantification of polyphenols and their use in pharmaceutical industries as a potential substitute of synthetic polyphenols.

1. Introduction

The health benefits of polyphenols to human beings have been attracted attention to them worldwide and these beneficial properties of polyphenols are generally accredited to their antioxidant nature [1]. Polyphenolic antioxidants (PA) are structural class of organic chemicals which contain phenol units and based on their origin PAs can be classified in two categories i) synthetic and ii) natural. Structurally, these are classified into i) Phenolic acids ii) Flavonoids iii) Lignans and iv) Stilbenes. Synthetic PAs such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tertiary-butylhydroquinone (TBHQ), octyl gallate (OG), dodecyl gallate (DG) etc. have been used in product formulations since 1950s in order to prevent or delay the onset of lipid oxidation during processing and storage of fats, oils and lipid containing foods [2]. Although these are effective in numerous food systems; yet their use

in the food industry is declining because of safety concerns and reports regarding the toxic effects of synthetic polyphenols are available [3, 4]. Amongst, BHA and BHT have observed susceptible in causing liver damage and carcinogenesis; also, BHA, TBHQ, PG are reported to cause damage in double helical structure of DNA [3, 5]. This has led interest of consumers towards natural products and the market for natural antioxidants is estimated 50% larger than it is for synthetics [6].

Naturally, in plants, PAs are among the most abundant secondary metabolites with approximately 8,000 known structures [7–10]. These are used in cosmetics and nutraceutical industries [11, 12] and due to their diverse uses and superiority over synthetic PAs, nowadays, studies are focusing on identifying natural sources of PAs which are low-cost and abundant in nature [13, 14].

The *Quercus* genus (family: Fagaceae) consists 500 species of trees and shrubs distributed mainly in Central America and Southeast Asia [15]. In the Himalayas, *Quercus* forms gregarious forest patches and sometimes grows in mixed forest formations with other broadleaved tree species, particularly in transition zones along the elevational gradient. *Quercus* species are rich in polyphenols and well-studied for their antiseptic, antidiarrheal, antimicrobial, anti-inflammatory, antioxidant, antitumoral, cytotoxic, gastroprotective, hemostatic, stomachic agent, and wound healing properties [16–18]. Leaves of *Quercus* species such as *Q. resinosa*, *Q. laeta*, *Q. grisea*, *Q. obtusata*, *Q. robur*, *Q. serrata* etc. are reported to have antioxidant and antimicrobial activity [19–22].

In plants, PAs occur in variable polarity states because of their diverse structural and physicochemical properties [23]. In food systems different interactions in between phenols and phenols with other constituents like acids, alkyl groups, sugars, etc. are reported emanating complex phenolic compounds such as polymeric phenols, condensed tannins, etc. [24]. Several methods such as enzymatic treatments, far-infrared radiation and heat treatments are known for the extraction of natural antioxidant polyphenols [25–28]. However, due to the variety in polyphenols and diverse nature of PAs, no single extraction technique is ideal for their optimum extraction [29, 30]. Traditional extraction methods used in extraction of PAs are energy and time consuming, also require larger solvent quantity that in some cases causes toxicity [31]. However, with the technological development, advanced extraction techniques and methods have been emerged for the improved and low-cost recovery of valuable PAs with comparatively less use of solvents, time, and energy [32, 33].

Optimization of extraction conditions is essential for economic commercial extraction process and can be achieved by considering several factors using empirical and/or statistical methods. Optimization factors, viz. solvent type and concentration, sample-to-solvent ratio, extraction time and extraction temperature etc., are known to influence the yield and quality of the desired compounds, and avoids chemical modifications [20, 34–36]. Response surface method (RSM) is reported effective in optimizing extraction procedures by investigating several influencing factors to the response and their interactions at one time with less experimental runs [37]. In recent studies, RSM has been used to optimize multifactor extraction conditions for polyphenolic compounds [19, 31, 37–40]. Based on the reviewed literatures, no optimal conditions are available for the extraction of PAs from Himalayan oak species. Thus, the present study envisaged to achieve optimum yield of PAs from leaves of Himalayan *Quercus* species through optimized heat assisted extraction (HAE) conditions.

2. Materials and methods

2.1. Plant material

In the month of June leaves from six Himalayan *Quercus* (oak) species viz. *Q. floribunda* Lindl. ex A. Camus (tilonj), *Q. franchetii* Skan (rianj), *Q. glauca* Thunb. (falant), *Q. serrata* Murrary

(tasar oak), *Q. oblongata* D. Don (banj) and *Q. semecarpifolia* Sm (kharsu) were collected from Uttarakhand, West Himalaya, India. Leaves were dried at room temperature until the constant weight achieved in successive weighing. Dried leaves were milled and stored at -20°C in separately sealed plastic bags; till further analysis.

Species identity was confirmed by consulting the available herbarium records of *Quercus* species at the departmental herbaria and authenticated by departmental taxonomist. The nomenclature of *Quercus* species follows the online source of The Plant List (<http://www.theplantlist.org>). The details of target species have been provided in S1 Table in S1 File.

2.2. Chemical and reagents

All High Performance Liquid Chromatography (HPLC) standards, and ascorbic acid; 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS); 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). However, solvents viz. ethanol, isopropanol and methanol were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Used HPLC standards were of HPLC grade and other chemicals and solvents were of analytical grade.

2.3. Solid-liquid extraction

Solvents of different polarities were examined for choosing appropriate solvent (Fig 1). Two gram leaf powder (sample) of *Q. semecarpifolia* was dissolved in 20 mL of 80% solvent (having remaining 20% distilled water) and kept at 60°C for heat assisted extraction in water-bath (Toshiba, India) for 60 min. Whatman paper (no 1) was used for phase separation. By considering the higher yield of total phenolic content (TP), best solvent was selected.

2.4. Experimental plan

The experimental plan followed a two-level multifactor design. Initially, a five factor [i.e., X_A : Extraction temperature ($^{\circ}\text{C}$), X_B : Extraction time (min), X_C : Solvent (methanol) concentration (%), X_D : Solvent pH, X_E : sample-to-solvent ratio (g/ml)] Plackett-Burman statistical design (PBD) involving 12 experimental runs considering only one dependent variable [Total Phenolic Content] was performed to select the influential factors (Table 1). In next step, most influencing three-factors [i.e., X_1 : Extraction temperature ($^{\circ}\text{C}$), X_2 : Solvent concentration (%) and X_3 : Sample-to-solvent ratio (g/ml)], were used in Box-Wilson Central Composite Design (CCD); linking 20 experiments considering five dependent variables [i.e. Total Phenolic Content, Total Flavonoid Content, Total Tannin Content, Total Antioxidant Activity (ABTS and DPPH)] (Table 2). Table 3 shows the operational conditions examined for individual dependent variable. PBD is mainly used to separate main contributing factors in extraction process and does not explain interactions between factors. In present study only those factors having confidence level $>95\%$ were considered. It was followed by Box-Behnken design (BBD) to screen process variables and their interactions on yield optimization of phytochemicals.

2.5. Analytical methods

2.5.1. The total phenolic content (TP). TP was calculated by following Folin-Ciocalteu's method described by Singleton and Rossi [41] and the content was quantified as milligram gallic acid equivalent per gram of dry leaf sample (mg GAE/g dw).

2.5.2. Total flavonoid content (TF). The Aluminium chloride (AlCl_3) method [42] was used for calculating total flavonoid content (TF) in *Quercus* leaf samples and quantified as milligram quercetin equivalent per of gram dry leaf sample (mg QE/g dw).

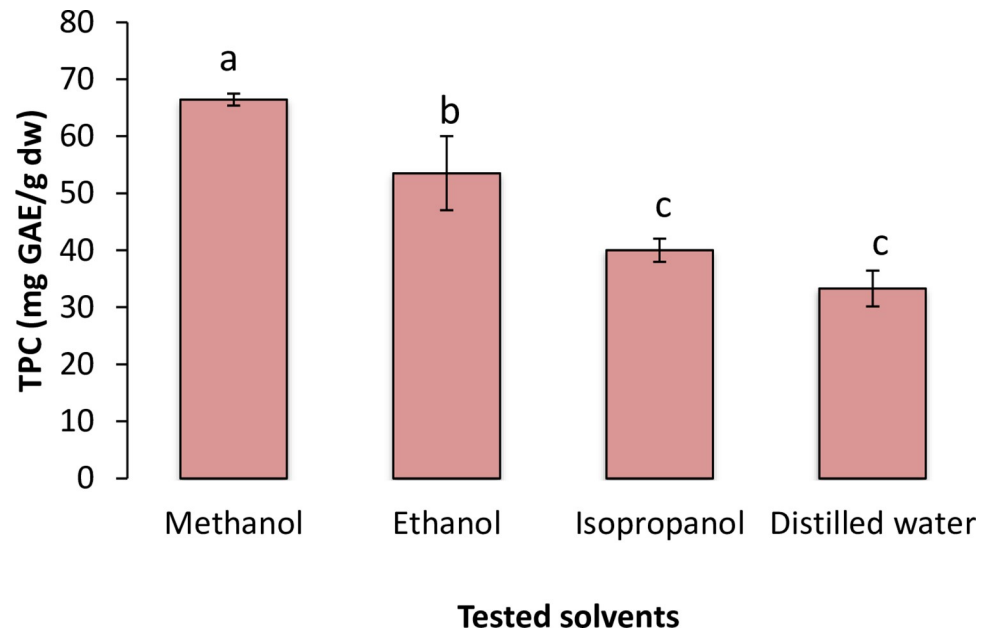


Fig 1. Solvent selection and response of different solvents on total polyphenolic content (TP). Bars capped with same letters are not significantly ($p < 0.05$) different to each other and separated using Duncan's Multiple Range Test (DMRT).

<https://doi.org/10.1371/journal.pone.0259350.g001>

2.5.3. Total tannin content (TT). The Folin-Denis colorimetric method was used to measure total tannin content (TT) by following Ram and Mehrotra [43], and results were expressed as milligram tannic acid equivalent per gram of dry leaf sample (mg TAE/g dw).

2.5.4. Total Antioxidant Activity (TAA). The antioxidant activity in *Quercus* leaf samples was analyzed through ABTS and DPPH assays by following Pandey et al. [19] and results were presented as millimolar ascorbic acid equivalent per gram of dry leaf sample (mM AAE/g dw).

Table 1. Plackett-Burman design (PBD) with responses of the dependent variables to extraction conditions.

| Experimental run | Independent variables | | | | | Dependent Variables |
|------------------|-----------------------|---------|---------|----------|-----------|---------------------|
| | X_A | X_B | X_C | X_D | X_E | TP(mg GAE/g dw) |
| 1 | 30 (-1) | 60(1) | 60 (1) | 6 (1) | 1:15 (-1) | 52.13 |
| 2 | 60 (1) | 30(-1) | 60 (1) | 2.5 (-1) | 1:15 (-1) | 76.77 |
| 3 | 30 (-1) | 30 (-1) | 60 (1) | 6 (1) | 1:30 (1) | 62.50 |
| 4 | 60 (1) | 60 (1) | 30 (-1) | 6 (1) | 1:30 (1) | 67.42 |
| 5 | 60 (1) | 60 (1) | 30 (-1) | 6 (1) | 1:15 (-1) | 58.88 |
| 6 | 60 (1) | 60 (1) | 60 (1) | 2.5 (-1) | 1:30 (1) | 83.26 |
| 7 | 30 (-1) | 30 (-1) | 30 (-1) | 6 (1) | 1:30 (1) | 53.62 |
| 8 | 60 (1) | 30 (-1) | 30 (-1) | 2.5 (-1) | 1:30 (1) | 62.26 |
| 9 | 60 (1) | 30 (-1) | 60 (1) | 6 (1) | 1:15 (-1) | 71.99 |
| 10 | 30 (-1) | 60 (1) | 60 (1) | 2.5 (-1) | 1:30 (1) | 67.70 |
| 11 | 30 (-1) | 60 (1) | 30 (-1) | 2.5 (-1) | 1:15 (-1) | 47.14 |
| 12 | 30 (-1) | 30 (-1) | 30 (-1) | 2.5 (-1) | 1:15 (-1) | 47.14 |

X_A = Extraction temperature ($^{\circ}$ C), X_B = Extraction time (min), X_C = Solvent concentration (%), X_D = pH, X_E = Sample-to-solvent ratio (g/ml), TP = Total polyphenolic content

<https://doi.org/10.1371/journal.pone.0259350.t001>

Table 2. Box-Wilson Central Composite Design (CCD) with responses of the dependent variables to extraction conditions.

| Experimental run | Independent variables | | | Dependent Variables | | | | |
|------------------|-----------------------|----------------|----------------|---------------------|-----------------|----------------|-------------------|-------------------|
| | X _A | X _C | X _E | TP(mg GAE/g dw) | TT(mg TAE/g dw) | TF(mg QE/g dw) | DPPH(mM AAE/g dw) | ABTS(mM AAE/g dw) |
| 1 | 60(0) | 70(0) | 1:30(0) | 64.66 | 67.54 | 27.89 | 25.44 | 1.78 |
| 2 | 40(-1) | 50(-1) | 1:40(+1) | 46.27 | 73.63 | 22.69 | 39.71 | 1.23 |
| 3 | 60(0) | 70(0) | 1:20(-1) | 48.74 | 44.29 | 18.65 | 18.46 | 1.26 |
| 4 | 60(0) | 90(+1) | 1:30(0) | 42.76 | 70.76 | 52.51 | 28.22 | 1.35 |
| 5 | 40(-1) | 90(+1) | 1:20(-1) | 32.78 | 41.84 | 37.82 | 19.37 | 1.02 |
| 6 | 80(+1) | 50(-1) | 1:20(-1) | 53.73 | 41.58 | 16.73 | 20.23 | 1.27 |
| 7 | 60(0) | 70(0) | 1:30(0) | 51.53 | 64.24 | 24.14 | 27.14 | 1.63 |
| 8 | 60(0) | 70(0) | 1:30(0) | 64.52 | 59.57 | 26.25 | 27.59 | 1.82 |
| 9 | 40(-1) | 90(+1) | 1:40(+1) | 31.77 | 76.31 | 46.41 | 38.58 | 0.94 |
| 10 | 60(0) | 70(0) | 1:30(0) | 47.53 | 64.52 | 30.77 | 25.79 | 1.76 |
| 11 | 60(0) | 70(0) | 1:30(0) | 49.58 | 59.88 | 30.10 | 26.35 | 1.77 |
| 12 | 80(+1) | 90(+1) | 1:40(+1) | 68.92 | 88.81 | 42.56 | 37.31 | 2.20 |
| 13 | 60(0) | 70(0) | 1:40(+1) | 64.96 | 84.30 | 30.38 | 40.42 | 1.96 |
| 14 | 40(-1) | 70(0) | 1:30(0) | 40.46 | 60.80 | 24.52 | 29.10 | 1.36 |
| 15 | 80(+1) | 70(0) | 1:30(0) | 91.27 | 61.52 | 31.45 | 31.19 | 1.89 |
| 16 | 60(0) | 50(-1) | 1:30(0) | 47.49 | 58.38 | 25.48 | 25.54 | 1.63 |
| 17 | 80(+1) | 90(+1) | 1:20(-1) | 65.42 | 42.43 | 33.33 | 17.86 | 1.26 |
| 18 | 60(0) | 70(0) | 1:30(0) | 49.32 | 62.17 | 31.06 | 25.44 | 1.73 |
| 19 | 80(+1) | 50(-1) | 1:40(+1) | 72.81 | 86.82 | 35.38 | 37.82 | 2.32 |
| 20 | 40(-1) | 50(-1) | 1:20(-1) | 49.01 | 42.66 | 15.83 | 18.88 | 1.23 |

X_A = Extraction temperature (°C), X_C = Solvent concentration (%), X_E = Sample-to-solvent ratio (g/ml)

TP = Total polyphenolic content, TT = Total tannin content, TF = Total flavonoids content, DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability, ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation inhibition

<https://doi.org/10.1371/journal.pone.0259350.t002>

2.6. HPLC analysis

The phenolic profiles of extracts were analyzed through high performance liquid chromatography (HPLC) (Shimadzu LC-10AT, Japan) coupled with diode array detector (DAD-MZOA)

Table 3. Analysis of variance (ANOVA) of the regression model from the Plackett-Burman Design (PBD) for major contribution to total phenol content.

| Source | Sum of Squares | DF | Mean Square | F value | P value |
|----------------|----------------|----|-------------|---------|-----------|
| Model | 1364.45 | 5 | 272.89 | 19.79 | 0.0011** |
| X _A | 680.13 | 1 | 680.13 | 49.33 | 0.0004*** |
| X _B | 0.42 | 1 | 0.42 | 0.03 | 0.8665 |
| X _C | 505.62 | 1 | 505.62 | 36.67 | 0.0009*** |
| X _D | 26.24 | 1 | 26.24 | 1.90 | 0.2169 |
| X _E | 152.03 | 1 | 152.03 | 11.03 | 0.0160* |
| Residual | 82.73 | 6 | 13.79 | | |
| Total | 1447.18 | 11 | | | |

X_A = Extraction temperature (°C), X_B = Extraction time (min), X_C = Solvent concentration (%), X_D = pH, X_E = Sample-to-solvent ratio (g/ml).DF = degrees of freedom.

Level of significance

* p < 0.05

** p < 0.01

*** p < 0.001.

<https://doi.org/10.1371/journal.pone.0259350.t003>

and two LC-10AT HPLC pumps [44]. Phenolic compounds were quantified by using peak area and prepared standard curve of corresponding phenolic compound standard. Each quantification of phenolic compounds was repeated three times for each *Quercus* species and expressed as milligram per gram dry weight of leaf samples (mg/g dw).

2.7. Statistical analysis

All analytical experiments were repeated three times, except RSM analysis, and obtained results were analyzed through analysis of variance (ANOVA) using SPSS statistical package for Windows (IBM SPSS Statistics 20, Chicago, USA). The individual and interrelated influence of significant factors on the extraction yield were studied by plotting three dimensional (3D) response surface plots through Design-Expert[®] version 12 software (Stat-Ease, Inc., MN, USA) and multiple regression analysis opted to analyze experimental data [45].

3. Result and discussion

3.1. Selection of solvents

Solvent type and solvent concentration are known to influence extraction yield and extraction of phytochemicals [7]. Four solvents were tested to determine the maximum TP content from *Quercus* leaves. Among all tested solvents (80% v/v), significantly ($p < 0.05$) higher yield of TP (66.43 mgGAE/g dw) was obtained in methanol followed by ethanol (53.51 mgGAE/g dw), isopropanol (40.02 mgGAE/g dw) and distilled water (33.30 mgGAE/g dw) (Fig 1). Thus methanol was used for further experiments.

3.2. Selection of significant factor

Plackett–Burman Design (PBD) was applied using five possible factors, namely, X_A : extraction temperature ($^{\circ}\text{C}$), X_B : extraction time (min), X_C : solvent concentration (%), X_D : solvent pH, X_E : sample-to-solvent ratio (g/ml) on TP concentration response. The analysis of variance (ANOVA) of the response data showed a significant effect of extraction temperature, solvent concentration, and sample-to-solvent ratio on TP response (Table 1). Overall, the PBD model was found significant ($p < 0.01$) with F-value of 19.79 and showed good determination coefficient value (0.94). All the significant factors showed positive regression coefficient value which revealed that the TP response increased with increase in extraction temperature, solvent concentration and sample-to-solvent ratio. Among these, the influence of extraction temperature on TP response was the maximum followed by solvent concentration and sample-to-solvent ratio (Fig 2). The remaining factors which includes, solvent pH (2) and extraction time (45 min) were kept at a constant value for further experiments.

3.3. Model fitness

Box–Wilson central composite design (BW-CCD) model was applied on significant factors over 3 levels to determine the linear, interactive and quadratic effect of factors on polyphenolic contents and antioxidant activity. To best fit the model in determining the optimized condition, model fitness analysis was conducted. All response models showed model fitness with the significant *F-value*, which indicates the significant role of factor's level in deterring the model variations. The coefficient of determination value was also found satisfactory for TP response, while for others it showed good R^2 value (Table 4). The insignificant lack of fit value was recorded for all responses except for DPPH. For all response models at least one factor has shown a significant linear, interactive and quadratic effect. As such, for TP response, the linear effect of extraction temperature was found significantly ($p < 0.001$) positive, while the solvent

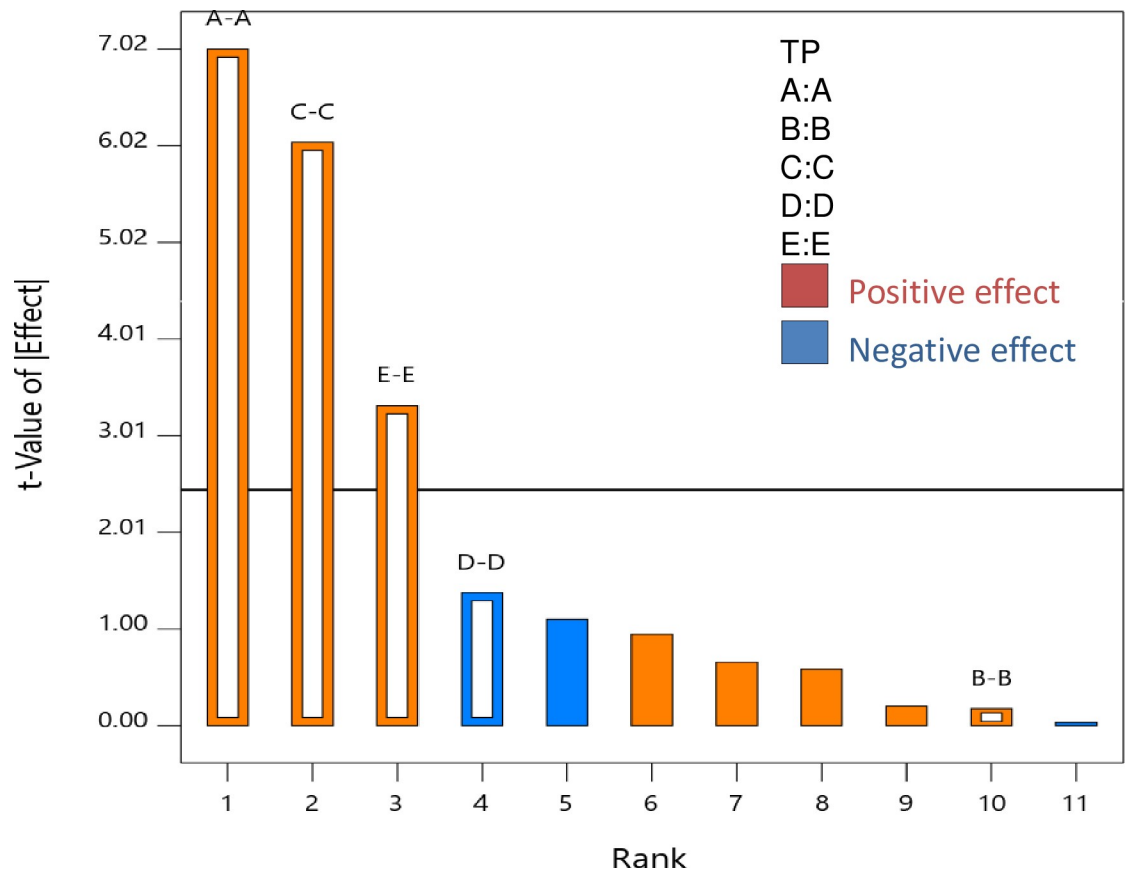


Fig 2. Pareto chart showing responses of the dependent variables to extraction conditions under Plackett-Burman design (PBD). A = Extraction temperature ($^{\circ}$ C), B = Extraction time (min), C = Solvent concentration (%), D = pH, E = Sample-to-solvent ratio (w/v), TP = Total polyphenolic content.

<https://doi.org/10.1371/journal.pone.0259350.g002>

concentration showed significant ($p < 0.05$) negative effect on TP. For TT response, a highly significant positive linear influence of extraction temperature ($p < 0.05$) as well as sample-to-solvent ratio ($p < 0.001$) was recorded. Moreover, a positive interactive effect between these factors has also affected TT response significantly ($p < 0.05$). Similarly, in case of TF, a highly significant ($p < 0.001$) positive linear effect of solvent concentration and sample-to-solvent ratio was recorded. Also, solvent concentration was found to produce a highly significant ($p < 0.001$) positive quadratic effect on TF. A significantly ($p < 0.05$) negative effect of sample-to-solvent ratio and an interactive effect between extraction temperature and solvent concentration was recorded for TF.

The antioxidant activity was analyzed using DPPH and ABTS *in vitro* assay. Both the assays were highly influenced by sample-to-solvent ratio and shown a significant ($p < 0.001$) positive linear effect (Fig 3E and 3F). Individually, ABTS activity showed a highly significant ($p < 0.001$) positive dependence on the extraction temperature. Also, a positive interactive effect of extraction temperature and solvent concentration was recorded for ABTS activity. However, significantly ($p < 0.01$) negative linear and quadratic effects of solvent concentration on ABTS activity were observed.

All the significant linear, quadratic and interactive term fitted to the second order polynomial equation as-

$$Y_{TP} = 55.42 + 15.19X_A - 11.65X_C^2$$

Table 4. Regression coefficient (β), coefficient of determination (R^2) and F-test value of the predicted second order polynomial models (CCD) for polyphenolics and antioxidant activities.

| Regression Coefficients (β) | | | | | |
|-------------------------------------|-----------------|-----------------|----------------|-------------------|-------------------|
| | TP(mg GAE/g dw) | TT(mg TAE/g dw) | TF(mg QE/g dw) | DPPH(mM AAE/g dw) | ABTS(mM AAE/g dw) |
| Intercept, X_0 | 55.42 | 63.32 | 28.86 | 27.04 | 1.72 |
| Linear | | | | | |
| X_A | 15.19*** | 2.59* | 1.22 | -0.1228 | 0.315*** |
| X_C | -2.77 | 1.71 | 9.65*** | -0.085 | -0.092** |
| X_E | 3.5 | 19.71*** | 5.51*** | 9.91*** | 0.2617*** |
| Quadratic | | | | | |
| X_A^2 | 9.08 | -2.66 | -1.63 | 1.98 | -0.0518 |
| X_C^2 | -11.65* | 0.7504 | 9.38*** | -1.29 | -0.1841** |
| X_E^2 | 0.0711 | 0.4773 | -5.09* | 1.27 | -0.0624 |
| Cross Product | | | | | |
| $X_A X_C$ | 4.82 | 0.12 | -2.74* | -0.2806 | 0.0472 |
| $X_A X_E$ | 3.29 | 3.27* | 1.55 | -0.375 | 0.2587*** |
| $X_C X_E$ | -1.73 | 0.5782 | -0.9615 | 0.0306 | -0.0222 |
| R^2 | 0.827098 | 0.973231 | 0.935638 | 0.972387 | 0.973269 |
| F value (model) | 5.32** | 40.4*** | 16.15*** | 39.13*** | 40.46*** |
| F value (lack of fit) | 1.18 | 1.41 | 1.83 | 5.97* | 2.33 |
| P value | 0.4296 | 0.3569 | 0.2612 | 0.0361 | 0.1879 |

X_A = Extraction temperature ($^{\circ}$ C), X_C = Solvent concentration (%), X_E = Sample-to-solvent ratio (g/ml), TP = Total polyphenolic content, TT = Total tannin content, TF = Total flavonoids content, DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability, ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation inhibition; Level of significance

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$.

<https://doi.org/10.1371/journal.pone.0259350.t004>

$$Y_{TT} = 66.32 + 2.59X_A + 19.71X_E + 3.27X_A X_C$$

$$Y_{TF} = 28.86 + 9.65X_C + 5.51X_E + 9.38X_C^2 - 5.09X_E^2 - 2.74X_A X_C$$

$$Y_{DPPH} = 27.04 + 9.91X_E$$

$$Y_{ABTS} = 1.72 + 0.315X_A - 0.092X_C + 0.2617X_E - 0.1841X_C^2 + 0.2587X_A X_E$$

3.4. Effects of factors on the responses

3.4.1. Effect of extraction temperature. Extraction temperature played a major role in polyphenolic contents as well as antioxidant activity from Oak leaves. Linear effect of extraction temperature significantly affected TP, TT and ABTS antioxidant activity. With an increase in extraction temperature from 40 $^{\circ}$ C to 80 $^{\circ}$ C, a highly significant ($p < 0.001$) linear increase of TP content was recorded (Fig 3A). Similarly, a significant ($p < 0.05$) increase in TT content was recorded with increasing temperature. However, the effect of extraction temperature was more on TP response compared to TT response (Fig 3B). Likewise, a highly significant ($p < 0.001$) linear increase in ABTS activity was seen with increasing extraction temperature and sample-to-solvent ratio (Fig 3F). A highly significant ($p < 0.001$) increase in ABTS activity was seen when both sample-to-solvent ratio and extraction temperature were raised simultaneously. Also, TT increased significantly ($p < 0.05$) with the increase in extraction temperature along with sample-to-solvent ratio (Fig 3B). Though, increasing solvent concentration and extraction temperature, has a significant ($p < 0.05$) negative effect on TF. Further, with decreasing extraction temperature and increase in solvent concentration, TF increased significantly

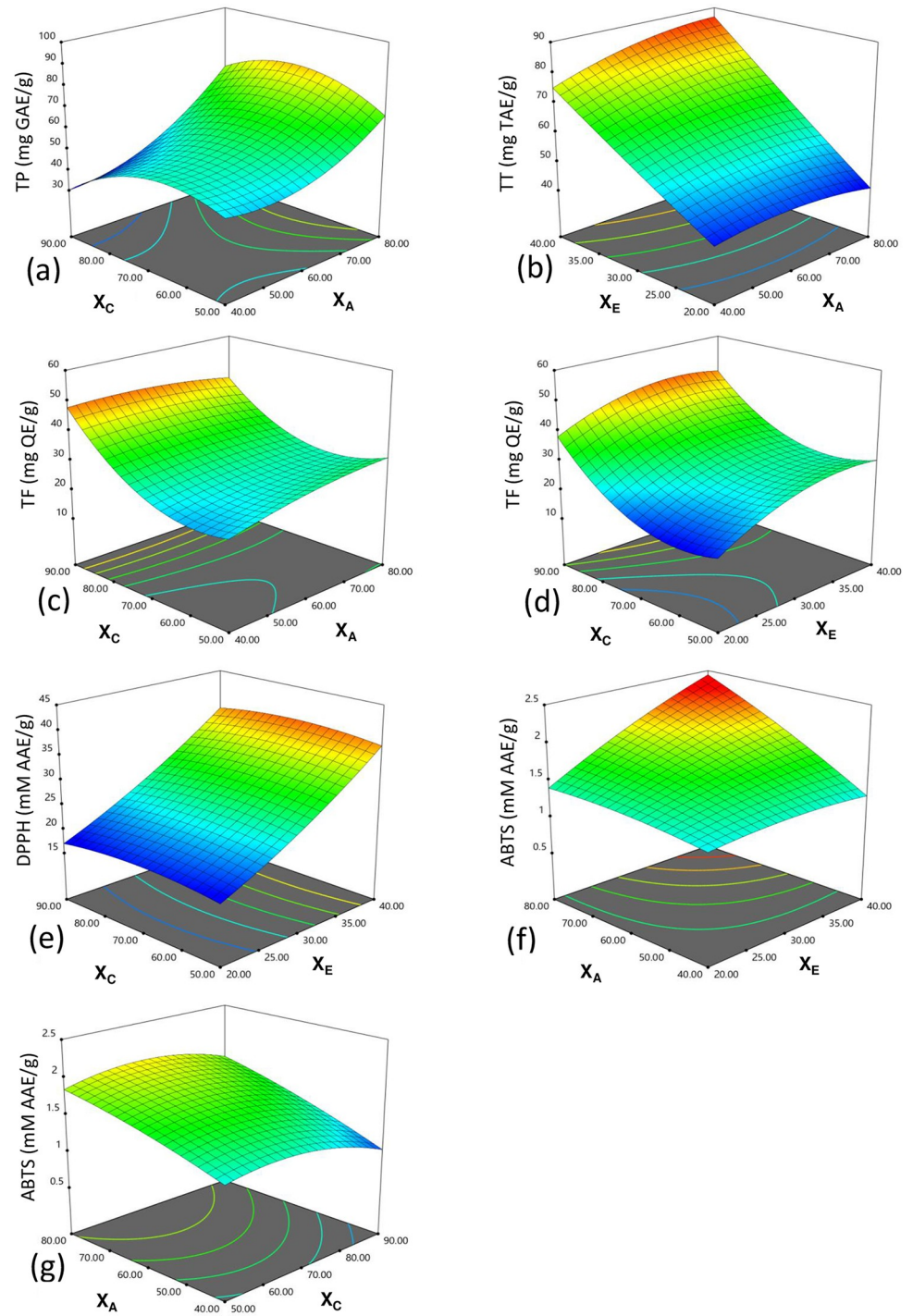


Fig 3. Response surface graphs showing the linear, quadratic, and interactive effect of different factors under Central Composite Design (CCD). X_A = Extraction temperature ($^{\circ}$ C), X_C = Solvent concentration (%), X_E = Sample-to-solvent ratio (g/ml), TP = Total polyphenolic content, TT = Total tannin content, TF = Total flavonoids content, DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability, ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation inhibition.

<https://doi.org/10.1371/journal.pone.0259350.g003>

(Fig 3C). The heat energy known to improve the effectiveness of extraction by distorting cellular structures, increasing the permeability of cell membrane and breaking-down of polyphenol-lipoprotein interactions, which leads to increased solubility and mass transfer of PAs into the solvent [46]. At high temperature the viscosity of extraction medium decreases, that helps solvents to enter the plant matrix leading to rapid kinetics [47]. Further, with the increase of solvent temperature surface tension decreases, that may increase wetting of the plant-particles, and resulting a higher extraction yield from softened plant tissue [48].

3.4.2. Effect of solvent concentration. With an increase in solvent concentration from 50% to 90%, a highly significant ($p < 0.001$) increase in TF concentration was recorded (Fig 3C). At higher solvent concentration, the TF increased profoundly as compared to the linear increase and showed highly significant ($p < 0.001$) positive quadratic effect. However, ABTS activity was observed decreasing with increasing solvent concentration (Fig 3G). Similarly, with increase in solvent concentration at higher level, TP significantly ($p < 0.05$) decreased. Also, with increase in extraction temperature the positive influence of solvent concentration on TF was found to be decreased and revealed a negative interactive effect (Fig 3C). Studies have indicated the efficacy of methanol over other organic solvents in phytochemical extraction [49, 50].

3.4.3. Effect of sample-to-solvent ratio. A highly significant ($p < 0.001$) positive linear effect of sample-to-solvent ratio was found on all responses except TP (Table 4 and Fig 3). Increasing sample-to-solvent ratio from 1:20 to 1:40 (w/v), a significant linear enhancement in TF, TT, ABTS and DPPH was recorded. It can be correlated to the hindrance of saturation in extraction medium by using higher volume of solvent [51, 52]. Also, with increasing extraction temperature and sample-to-solvent ratio, the TT and ABTS values were increased significantly. Optimized sample-to-solvent ratio is essential to develop equilibrium between high extraction costs and wastage of solvents and deterrence of saturation effects [53]. It is reported that some polyphenolic compounds possess complex structures that are soluble only in organic solvents and their combinations often with different proportions of water [7]. Therefore, increasing methanol concentration may enhance the extraction of soluble polyphenolic compounds from plant samples. Furthermore, being the high polarity solvent, methanol improves the release of bioactive compound and may serve as a green solvent for extraction of PAs [54]. According to the study by Capello et al. [50] on comprehensive framework for the environmental assessment of solvents, among tested 26 organic solvents in order to identify green solvents, methanol-water or ethanol-water mixtures were emerged environmentally benign compared to pure alcohol or propanol-water mixtures.

3.5. Optimized HAE condition and its validation

Based on the above experiments (section 3.1–3.4), the optimized heat assisted extraction (HAE) condition for *Q. semecarpifolia* was determined as: temperature (80°C), solvent concentration (87% methanol), sample-to-solvent ratio (1: 40), solvent pH (2), and extraction time (45 min). The optimized condition was further tested on five *Quercus* species i.e. *Q. floribunda*, *Q. franchetii*, *Q. glauca*, *Q. oblongata*, and *Q. serrata*. The TP was recorded maximum (77.34 mg GAE/g dw) in *Q. semecarpifolia* and minimum (63.01 mg GAE/g dw) in *Q. serrata*. However, TT (94.19 mg TAE/g dw) and TF (87.05 mg QE/g dw) content were observed maximum in *Q. serrata* and minimum TT (80.10 mg TAE/g dw) in *Q. franchetii* and TF (27.24 mg QE/g dw) in *Q. glauca* respectively (Table 5). All values were found close to the model's predicted value which indicates that the model is well-fit for the extraction of polyphenolic compounds from *Quercus* leaves under optimal HAE condition and the used RSM models are good for predicting the optimal extraction condition.

Table 5. Comparative phytochemical profile of six *Quercus* species under optimal extraction condition.

| Phytochemical screening | <i>Quercus</i> species | | | | | |
|--|--------------------------------|---------------------|----------------------|----------------------|--------------------------|-------------------|
| | <i>Q. glauca</i> | <i>Q. oblongata</i> | <i>Q. floribunda</i> | <i>Q. franchetii</i> | <i>Q. semecarpifolia</i> | <i>Q. serrata</i> |
| Phytochemicals and antioxidant activity | | | | | | |
| TP(mg GAE/g dw) [<i>pv</i> 78.44] | 65.04 | 74.85 | 67.97 | 70.70 | 77.34 | 94.19 |
| TT(mg TAE/g dw) [<i>pv</i> 89.29] | 84.72 | 86.42 | 90.23 | 80.10 | 91.45 | 87.05 |
| TF(mg QE/g dw) [<i>pv</i> 42.23] | 37.24 | 27.24 | 38.97 | 31.67 | 44.42 | 33.10 |
| DPPH(mM AAE/g dw) [<i>pv</i> 38.49] | 42.22 | 39.09 | 37.62 | 42.51 | 35.35 | 2.18 |
| ABTS(mM AAE/g dw) [<i>pv</i> 2.25] | 2.16 | 2.14 | 2.15 | 2.14 | 2.10 | 63.01 |
| Phenolic compounds | | | | | | |
| | Concentration (mg/g dw) | | | | | |
| 3-Hydroxy Benzoic acid | 3.69±0.04 | 1.14±0.31 | 2.24±0.12 | 1.96±0.15 | - | 1.95±0.23 |
| Caffeic acid | - | - | - | - | 0.09±0.00 | 0.03±0.00 |
| Catechin | - | - | 37.60±0.65 | 2.20±0.28 | 28.31±0.00 | 19.53±0.58 |
| Chlorogenic acid | 5.22±0.08 | 6.21±0.12 | - | 1.30±0.17 | - | - |
| Ellagic acid | - | - | - | 0.10±0.00 | - | - |
| Ferulic acid | - | 0.23±0.00 | - | 0.15±0.02 | - | 0.129±0.00 |
| Galic acid | 23.45±0.19 | 28.28±0.32 | 14.81±0.18 | 21.55±1.41 | 16.53±0.24 | 17.28±0.06 |
| Rutin | - | - | - | - | 3.51±0.03 | 3.52±0.03 |
| Trans cinnamic acid | 0.04±0.00 | 0.10±0.00 | - | 0.01±0.00 | - | - |
| Vanillic acid | 0.44±0.01 | 0.60±0.05 | - | 0.25±0.01 | 0.16±0.01 | 0.44±0.02 |
| m-coumaric acid | 0.25±0.01 | - | - | - | - | - |
| p-coumaric acid | - | - | - | - | 0.10±0.01 | 0.11±0.00 |

TP = Total polyphenolic content, TT = Total tannin content, TF = Total flavonoids content, DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability,

ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation inhibition

Pv = model's predicted value; - = not detected

<https://doi.org/10.1371/journal.pone.0259350.t005>

3.6. Polyphenolic screening

Under optimum HAE condition, the HPLC analysis revealed presence of 12 antioxidant polyphenolic compounds (caffeic acid, catechin hydrate, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, rutin hydrate, trans cinnamic acid, *m*-coumaric acid, *p*-coumaric acid, 3-hydroxybenzoic acid and vanillic acid) in the leaf extracts of total screened (six) *Quercus* species, described in section 3.5 (Table 5). The polyphenolic compound composition and concentration varied in target species. *Q. franchetii* and *Q. serrata* were among the highest (8 in each) polyphenolic compound containing species followed by *Q. glauca*, *Q. oblongata*, *Q. semecarpifolia* (6 each) and *Q. floribunda* (3). Among the detected compounds, catechin was found in highest concentrations in *Q. floribunda* (37.60 mg/g leaf dw), *Q. semecarpifolia* (28.31 mg/g leaf dw) and *Q. serrata* (19.53 mg/g leaf dw) respectively. Followed by gallic acid, and it was detected maximum in *Q. oblongata* (28.276 mg/g leaf dw), *Q. glauca* (23.45mg/g leaf dw), and *Q. franchetii* (21.55 mg/g leaf dw) respectively (Table 5). The quantified polyphenolic compounds from six Himalayan *Quercus* species such as rutin, *p*-coumaric acid, catechin, gallic acid, 3-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, ellagic acid, *m*-cinnamic acid, *p*-cinnamic acid and chlorogenic acid have gained significant attention due to their multiple biological activities including antioxidant, antimicrobial, anticancer, antidiabetic, anti-inflammatory, antihepatotoxic, anticholestatic, antihepatocarcinogenic antisteatotic, free radical scavenging, cytotoxic, gastric protective and inhibition of HIV replication [55–58]. The highest recorded phenolic compounds in the current studied *Quercus* species *i.e.* catechin and gallic acid are known to present in many dietary products, plants, fruits, etc. and clinical studies have shown the beneficial effects of catechin and gallic acid due their antioxidant action

[59]. Catechin plays a significant role in several molecular mechanisms such as angiogenesis, degradation of extracellular matrix, regulation of cell death, and multidrug resistance in cancer and associated disorders [59]. Reports indicate that the PAs in studied Himalayan *Quercus* species are comparable to various medicinal plants of Himalayan region studied in same laboratory conditions such as 12 PAs in [*Rheum moorcroftianum* under ultrasonic assisted extraction [19]; 6, 7 PAs in *B. jaeschkeana* and *B. asiatica* respectively under microwave assisted extraction [43], 13 PAs in different *in vitro* growing stages of *Origanum vulgare* under heat assisted extraction [44]; 10 and 9 PAs under microwave assisted extraction and ultrasonic assisted extraction respectively in *Berberis jaeschkeana* [60]. Furthermore, this is the first report on extraction optimization of twelve antioxidant polyphenolic compounds in a single extraction process from Himalayan *Quercus* species. Considering the significant extraction yield of polyphenolic compounds such as gallic acid, catechin, chlorogenic acid, etc. this optimized HAE condition can be extended to the industrial purpose.

4. Conclusion

Among six *Quercus* species examined in the present study, *Q. semecarpifolia* and *Q. serrata* exhibited significantly higher levels of polyphenols under the optimized extraction condition i.e. extraction temperature (80°C), solvent concentration of (87%; 0.2N methanol), sample-to-solvent ratio (1: 40), solvent pH (2), and extraction time (45 min). Gallic acid was observed in all the tested *Quercus* species however, highest catechin concentration was observed for *Q. floribunda*. *Quercus glauca* and *Q. franchetii* had significantly stronger DPPH radical scavenging activity or reducing power compared with that of the other tested species. No significant difference was observed in ABTS radical scavenging activity of tested six *Quercus* species. This study indicates that all the tested *Quercus* species of central Himalaya have a high utilization potential because of their high phenolic and flavonoid contents as well as strong antioxidant nature. Further, presence of significant catechin concentration in leaves of these species indicates use of *Quercus* species especially *Q. floribunda*, *Q. semecarpifolia* and *Q. serrata* in nutraceutical interventions and the extract after evaporating the solvent can be used in many nutrient formulations like beverages, dietary products etc. Use of *Quercus* species in pharmaceuticals and nutraceuticals may be helpful in reducing the extraction pressure from the threatened medicinal plants of Himalaya.

Supporting information

S1 File.
(DOCX)

Acknowledgments

Authors are thankful to the Director of G.B. Pant National Institute of Himalayan Environment (GBPNIHE) for providing necessary facilities. AP and ST are indebted to the Integrated Eco-Development Research Programme (IERP) cell of GBPNIHE for providing financial support. The financial support from International Centre for Integrated Mountain Development (ICIMOD), Nepal to AP through Khangchendzonga Landscape Conservation and Development Initiative (KLCDI)-India program is gratefully acknowledged.

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