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Griffith's Plum Yew (*Cephalotaxus griffithii* Hook. f.)- A gymnosperm from Kumaun Himalaya: As a source of total phenolic and total flavonoid compound, Antioxidant potential and Antimicrobial agents

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Abstract

Phytochemicals in medicinal plants have received a great deal of attention mainly on their role in preventing diseases caused by microbial attack and oxidative stress.

The present study aimed to evaluate the biochemical properties of Griffith's plum Yew *Cephalotaxus griffithii* Hook. f. needles using two different extracts (hexane and aqueous) by qualitative and quantitative phytochemical methods along with their antibacterial activity against some gram-positive and gram-negative bacteria responsible for many diseases including human.

From the observed results it is clear that aqueous extract was found more suitable to extract out the primary as well as secondary metabolites but hexane was found better for secondary metabolites extraction and also showed good amount of total phenolic and total flavonoid content ranged from 210-44 mg/ gm dry extract weight of the plant with low IC_{50} value for free radical scavenging activity.

In antimicrobial screening the hexane extracts was found effective against only gram positive bacteria tested with zone of inhibition (ZOI) upto 14 mm at 1000 µg/ml concentration. These results reveal that the extracts of *C. griffithii* is a possible good source of phenolic and flavonoid content as well as good free radical scavenger along with having good antimicrobial potential and new antibiotics.

Keywords: *Cephalotaxus griffithii, phytochemical, phenol, flavonoid, antioxidant, antimicrobial.*

I. Inroduction

Many plant species found in Himalaya area well known for their medicinal value for a long time. The importance of medicinal plants is being highlighted as a source of natural antioxidant and functional foods [1]. The antioxidant property of the plants considered the best as it reduces the oxidation processes in the living system [2] and plays an important role in maintaining health by reducing harmful reactive oxygen species (ROS) [3]. In recent years, phytochemicals in medicinal plants have received attention on their role in preventing diseases caused due to oxidative stress which releases ROS such as singlet oxygen and various radicals as a damaging side-effect of aerobic metabolism. These radicals are possibly involved in a number of disorders including cardiovascular malfunctions, tissue injury, DNA damage, tumor promotion, kidney and liver disease,

fibrosis, atherosclerosis, arthritis, neurodegenerative disorders and aging. Several studies suggested that antioxidants could prevent accumulation of these reactive oxygen species and beneficial for treatment of these pathologies [4,5] Many research studies have demonstrated that medicinal plants, fruits and vegetables contain various components with antioxidant activity, which are responsible for their beneficial health effects. Due to their natural origin, the antioxidants obtained from plants are of greater benefit in comparison to synthetic ones.

Further, antimicrobials of plant origin have enormous therapeutic potential [6]. Human infections particularly those involving microorganisms *i.e.* bacteria, fungi, viruses, cause serious infections in tropical and subtropical countries of the world. In recent years, multiple drug resistance in human pathogenic microorganisms has been developed due to indiscriminate use of commercial antimicrobial drugs [7]. Over the past twenty years, there has been a lot of interest in the investigation of natural materials as sources of new antimicrobial agents [8]. The use of plants extracts by extracting their phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. [9-13].

Cephalotaxus griffithii Hook.f. is a shrub or small tree and grows at altitude of 2000 m. This plant is distributed in Asia [14] and endemic to North east India, especially in Mishmi Hills, Assam to Northern India, western Sichuan Provience in China, Myanmar, Japan and Thailand [15, 16]. The needles are 2-3 inch long and 3 mm wide. Bark is blackish green. The plant is traditionally used in Manipur, India in the treatment of cancer [17]. The major component of its volatile oil are α-pinene, caryophyllene, β-pinene, mytcene and limonene [18].

II. MATERIAL AND METHODS

Sample collection

Leaves (needles) of selected plant was collected during the month of March 2016, from district Nainital (Uttarakhand), India and authenticated by Prof. Y. P. S. Pangtey, Department of Botany of the Kumaun University. A voucher specimen was deposited in the departmental herbarium.

Solvent Extraction of Plant Material

Plant material was thoroughly washed off under running tap water and then shade dried at room temperature for three-four weeks. The air-dried plant material was finely grinded and packed in self seal air tight polythene bags for further use. Exposure to sunlight was avoided to prevent the loss of active components.

Fine powdered plant material was soaked in two different solvents (1:10 w/v) separately *i.e.* Hexane (highly non-polar) and double distilled water (*i.e.* aqueous) (highly polar) for 48 hours in closed electrical shaker (120 rpm at 25ºC) and then filtered with Whatman's filter paper no.1. Only supernatant was taken and solvent was evaporated using vacuum evaporator at 40^0C and stored at 4ºC for further studies.

Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), quercetin, sodium nitrite ($NaNO_2$), ascorbic acid, Ferric chloride ($FeCl_3$), gallic acid, sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), potassium per-sulfate ($K_2S_2O_8$), Ethylene diamine tetra acetic acid (EDTA), were obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. Folin-Ciocalteu's reagent, Molisch's reagent, conc. H_2SO_4, Fehling's reagent, glacial acetic acid, conc. HCl, NH_4OH, Meyer's reagent (potassiomercuric iodide solution), 2,2 [azinobis (3-ethyl benothiazoline- 6-sulphonic acid) diammonium salt] (ABTS), hexane, chloroform, ethanol and. methanol were obtained from Merck, Mumbai, India. All chemicals used were of analytical grade.

Media Used : Nutrient agar (NA), Nutrient Broth (NB), Muller Hinton Agar (MHA), Brain Heart Infusion Agar (BHIA), Brain Heart Infusion Broth (BHIB) (Hi Media Laboratories Ltd., Bombay).

QUALITATIVE PHYTOCHEMICAL SCREENING

To assess the chemical composition of the plant qualitatively, phytochemical analysis was conducted using hexane and aqueous extracts following Harborne, 1998 [19].

QUANTITATIVE PHYTOCHEMICAL ASSAYS

Determination of Total Phenolic Content (TPC)

The total phenolic content of the sample extract was determined by Folin-Ciocalteu's colorimetric method given by Singleton and Rossi [20] with certain modifications. Absorbance of the sample was measured spectrophotometrically (UV-VIS) at 765nm. Quantification of total phenolic content was based on standard curve of Gallic acid. The results were expressed in mg gallic acid equivalent (GAE)/gm dry extract weight of the sample.

Determination of Total Flavonoid Content (TFC)

Content of flavonoids of the sample extract were determined by $AlCl_3$ colorimetric method given by Chang *et al*, [21] with certain modifications. The absorbance was recorded at 415 nm using UV-VIS spectrophotometer. Quantification of total flavonoid content was done on the basis of standard curve of Quercetin. Results were expressed in mg quercetin equivalent (QE)/gm dry extract weight of the sample.

DETERMINATION OF ANTIOXIDANT ACTIVITY

The antioxidant potential of *C. griffithii* was evaluated by two different methods, DPPH (1,1-diphenyl 2-picrylhydrazyl), and ABTS (2,2' [azinobis (3 ethyl benothiazoline- 6 sulphonic acid).

DPPH Antioxidant Activity Assay

The DPPH assay was done according to the method of Brand-Williams *et al*. (1995) [22] with certain modifications. The reduction in absorbance was recorded at 517 nm in UV-VIS spectrophotometer. Ascorbic acid (AA), Butylated hydroxyl anisole (BHA) and Butylated hydroxyl toluene (BHT) was used as standard and for control

absorbance of DPPH cations was taken without adding sample extract.

ABTS Antioxidant Activity Assay

The ABTS (2,2' -Azinobis-3-ethylbenzotiazoline-6-sulphonic acid) assay was conducted according to [23, 24] Miller *et al*. (1993) and Re *et al.* (1999) with minor modifications. The antioxidant activity of tested sample was calculated by determining the decrease in the absorbance at different concentrations.

% scavenging /Inhibition for DPPH, and ABTS were calculated as:

$$\% \text{ scavenging} = \frac{\text{absorbance of control} - \text{absorbance of test sample}}{\text{absorbance of control}} X100$$

ANTIMICROBIAL ACTIVITY

Microorganisms used maintaining culture media

The bacterial culture used were obtained from IMTECH, Chandigarh. Four microorganisms *Proteus mirabilis* (MTCC 3310), *Klebsiella pneumoniae* (MTCC 7028), *Pseudomonas aeruginosa* (MTCC 3542) and *Staphylococcus aureus* (MTCC 737) were used to test the sensitivity against plant extracts. Bacterial stains were revived and grown on nutrient agar plates at 37ºC and maintained by periodic subculture on nutrient agar plates.

Preparation of standard culture inoculums of test organism

Three or four isolated colonies were inoculated in the 25 ml nutrient broth and brain heart infusion broth and incubated for 24 hour at 37ºC in shaking condition for even suspension of bacterial colonies and maintained equivalent with Mac-Farland standard (0.5%) as recommended by WHO.

Determination of zone of inhibition (ZOI) and minimum inhibitory concentration (MIC)

Antibacterial tests of selected microorganisms were carried out by using disc diffusion method [25]**.** The freshly prepared inoculums was swabbed on MHA plates using sterile cotton swab and left the plates in laminar for 1 hour. Sterile discs were soaked with 40 µl of the different extracts of selected plants with the help of micropipette. Plates were incubated with closed lid at 37ºC and 30ºC for 24 hour and measure the zone of inhibition with the help of zone meter. All the tests were performed in triplicates and mean of triplicates was considered as MIC of the extract tested.

Statistical Analysis

All the measurements were taken in triplicates and the results obtained were expressed as mean ± standard error (SE). The results were further analyzed by ANOVA (analysis of variance) and Duncan test using SPSS 20.0 software. At $p<0.05$ and $p<0.01$ the values were considered to be significant.

III. RESULTS

The qualitative phytochemical analysis for the presence of 13 phytochemical groups, quantitative phytochemical analysis to estimate the total phenolic content (TPC) and total flavonoid content (TFC) and antioxidant potential evaluation using two different

methods (DPPH and ABTS assays) were carried out using two extracts, hexane- CG_H and aqueous- CG_A extracts of *Cephalotaxus griffithii* needles

PHYTOCHEMICAL ANALYSIS

Qualitative Phytochemical Analysis

Phytochemical analysis of the prepared extracts (CG_H and CG_A) revealed the higher number of phytochemicals were extracted in polar extract *i.e*. aqueous CG_A (7) than the non-polar hexane extract CG_H (5). Phenols, flavonoids, and saponins were tested positive in both the fractions used while carbohydrates and proteins were found only in CG_A (Table 1).

Table 1: Qualitative Phytochemical Analysis of different extracts of *C. griffithii* needles

S. N.	Phytochemicals	Tests performed	Solvent extracts	
			CG_H	CG_A
1.	Carbohydrates	Molish's test	-	+
		Fehling's test	-	-
2.	Protein	Biuret test	-	+
		Millon's test	-	-
		Xanthoprotic test	-	-
3.	Alkaloids	Mayer test	+	-
		Wagner test	-	-
4.	Flavonoids	Ferric chloride test	+	-
		NaOH Test	-	+
5.	Phenols	$FeCl_3$ Test	+	+
6.	Tannin	$Fecl_3$ Test	-	+
7.	Gallo-tannin	$FeCl_3$ Test	-	-
8.	Resin	Turbidity Test	-	+
9.	Saponin	Foam Test	+	+
10.	Quinones	H_2SO_4 Test	-	-
11.	Volatile oils	NaOH-HCl Test	-	-
12.	Glycosides	Keller-Kiliani Test	+	-
13.	Terpenoids	Salkowski's Test	-	-
	Total		**5**	**7**

+ = Present; - = absent; CG_H – *C. griffithii* hexane extract; CG_A – *C. griffithii* aqueous extract

Quantitative Phytochemical Analysis

Determination of Total Phenolic Content (TPC)

Result of total phenolic content experiments were calculated using regression equation of standards (GA, BHA and BHT). Table 2 shows highest total phenolic content in CG_H in comparison with CG_A.

Determination of Total Flavonoid Content (TFC)

The total flavonoid content was calculated by using regression equation of three standards (Quercetin, BHA and BHT). Results reveals that highest amount of TFC was found in CG_H and lowest amount was found in CG_A (Table 2).

Table 2: Total phenolic content (TPC) and Total flavonoid content (TFC) in different extracts of *C. griffithii* needles with respect to Quercetin, BHA and BHT

S. N.	Extracts	TPC			TFC		
		mg GAE gm^{-1} dry extract	*mg BHAE gm^{-1} dry extract*	*mg BHTE gm^{-1} dry extract*	*(mg QE gm^{-1} of dry extract*	*mg BHAE gm^{-1} of dry extract*	*mg BHTE gm^{-1} of dry extract*
1.	CG_H	210.12±4.12	112.5±7.22	85.71±5.50	86.21±7.18	127.78±11.57	140±13.88
2.	CG_C	197.62±3.15	89.58±5.51	68.58±4.20	36.78±5.01	48.15±8.07	44.44±9.69

Values are mean±SE of three independent observations, each in triplicate;
GAE- Gallic Acid Equivalent; QE - Quercetin Equivalent; BHAE- Butylated Hydroxy anisole Equivalent; BHTE - Butylated Hydroxy toluene Equivalent

DETERMINATION OF ANTIOXIDANT ACTIVITY

DPPH Free Radical Scavenging Activity Assay

DPPH free radical scavenging activities of plant extracts were analyzed using different concentrations at definite interval (1-50 µg ml^{-1}). At highest concentration used (50 µg ml^{-1}), CG_H showed higher inhibition of DPPH free radicals with 36.34 % inhibition (IC_{50}- 81.30±2.59 µg ml^{-1}) than CG_A with 32.7 % inhibition (IC_{50}- 82.40±2.07 µg ml^{-1}) (Table 3).

ABTS Free Radical Scavenging Activity Assay

The ABTS scavenging activities of different extracts were measured using three different standards (AA, BHA, and BHT). Highest ABTS radicals scavenging (44.95 %) was exhibited by CG_H with (39% inhibition; IC_{50}- 72.55±10.45 µg ml^{-1}) than CG_A with 34% inhibition (IC_{50}- 134.74±33.77 µg ml^{-1}) (Table 3).

Table 3: % Inhibition of DPPH and ABTS radicals at 50 µg ml^{-1} and IC_{50} values of different extracts of *C. griffithii* needles and standards

S.N.	Samples	DPPH		ABTS	
		% inhibition	*IC_{50} (µg ml^{-1})*	*% inhibition*	*IC_{50} (µg ml^{-1})*
1.	AA	72.98	6.42±0.61	99.12	9.08±2.12
2.	BHA	79.05	3.48±0.74	96.64	12.77±0.83
3.	BHT	68.57	8.40±1.31	98.94	6.05±0.92
4.	CG_H	36.34	81.30±2.59	39.36	72.55±10.45
5.	CG_A	32.70	82.40±2.07	34.22	134.74±33.77

Values are mean±SE of three independent observations, each in triplicate;
AA- Ascorbic Acid; BHA- Butylated Hydroxy anisole; BHT - Butylated Hydroxy toluene

DETERMINATION OF ANTIMICROBIAL POTENTIAL

Antimicrobial Activity Screening

The *in-vitro* antimicrobial screening of two extracts of *C. griffithii* was observed by the presence or absence of zone of inhibition with the help of disc diffusion method. Results are summarized in Table 4, reveals that CG_H showed activity against two bacteria *S. aureus* (ZOI 14 mm), and *L. monocytogenes* (ZOI 12.5 mm), whereas CG_A was found completely inactive in all cases.

Minimum Inhibitory Concentration (MIC)

Out of total 10, cases only 2 cases were found active in screening test showing positive activity against tested bacteria were further tested to evaluate minimum inhibitory concentration (MIC). The MIC values recorded for different bacteria with corresponding ZOI, and are summarized in Table 5. The MIC value against both the sensitive strains of CG_H was 125 µg ml^{-1} with ZOI 7.3±0.17 mm for *S. aureus* and 6.17±0.17 mm for *L. monocytogenes*.

Table 4: Antibacterial screening of different extracts of *C. griffithii* needles and different standards

Bacterial Strains	ZOI (mm)				
	1000 µg ml^{-1} concentration		*10 mcg*		*30 mcg*
	CG_H	CG_A	G	A	K
P. aeruginosa	NA	NA	14	NA	12
K. pneumoniae	NA	NA	15	13	14
P. mirabilis	NA	NA	17	NA	12
S. aureus	14	NA	19	NA	16.5
L. monocytogenes	12.5	NA	20	12.5	15.5

NA- Not active; G- Gentamicin; A- Ampicillin; K- Kanamycin

Table 5: MIC of hexane extract of *C. griffithii* needles along with corresponding ZOI (mm)

Bacterial Strains	CG_H	
	MIC (µg ml^{-1})	*ZOI (mm)*
P. aeruginosa	NT	NT
K. pneumoniae	NT	NT
P. mirabilis	NT	NT
S. aureus	125	7.3±0.17
L. monocytogenes	125	6.17±0.17

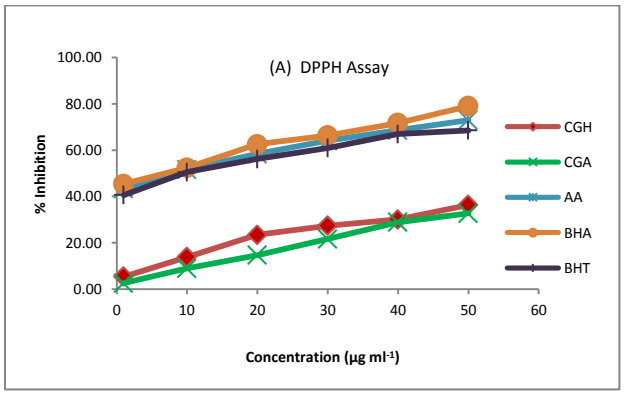
NT- not tested due to lake of observable inhibition at 1000 µg $ml^{-1;}$ Values are mean±SE of three independent observations, each in triplicate.

IV. DISCUSSION

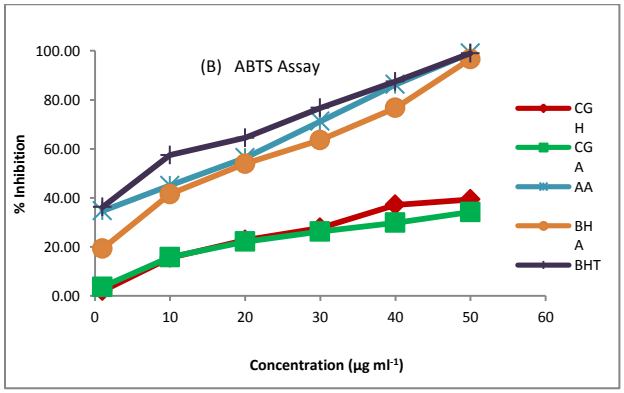
Out of 11 species of *Cephalotaxus* found in Northern hemisphere, literature survey reveals that *C. griffithii* is less investigated species [15]. Kamil *et al*. [26] isolated and characterized six flavonoids from *C. griffithii* needles, Phutdhawong *et al*. [18] reported the chemical profiling of essential oil of *C. griffithii*. Recently this species received interest for evaluation of different properties of the different plant parts including chemical profiling [17, 27-28], but extracts of needles of the plant has remained unexplored for its phytochemical characterization, antioxidant and antimicrobial activity as well. Although few other species namely *C. hainanesis* [29,30], *C. oliveri* [31], C *harringtonia* [32], *C. koreana* [33, 34 has been evaluated for their different activities as well as chemical profiling.

Number of phytochemicals present were higher in CG_A (7) in comparison with CG_H (5). The trend indicated relatively greater yield of substances in polar solvent. These primary and secondary phytochemicals which are synthesized through different pathways play vital role in plants health as well as provide medicinal value to it. From the present investigation it is clear that aqueous can be used to extract primary metabolites, while organic solvents are useful in extracting secondary metabolites.

Total phenolic content and antioxidant activity in DPPH assay have demonstrated a direct and positive correlation, which was evaluated in the samples on the basis of their abilities to reduce free radicals. Greater the absorbance value, superior is the reducing power of the extract, and perusal of Fig 1 clearly suggest a concentration dependent activity of the extracts. Between the two selected extracts CG_H is found to have greater ability in scavenging free radicals as opposed to CG_A fraction, the latter being the weak. Although the antioxidant capacities correlated linearly with the total phenol content, however the difference in the total flavonoid content and ABTS assay point to the fact that the antioxidant activity in this scavenging assay were a resultant of the effects of flavonoids in addition to the phenolic content.



[A]



[B]

Fig 1: Free radical Scavenging activities of two different extracts of *C. griffithii* needles and standards using (A) DPPH Assay (B) ABTS Assay.

In an another study done on antioxidant activity of bark acetone extract of *C. griffithii*, was reported to have a high antioxidant activity [17]. In present analysis, highest antioxidant activity was recorded for CG_H. This is interesting as acetone and chloroform have almost similar polarity. This observation support previous finding which highlight the importance of using a range of solvents for extraction purpose [35]. Fig 2, illustrates the high antioxidant potential of compounds having affinity for hexane fraction with low IC_{50} value, which is supported with high content of TPC and TFC.

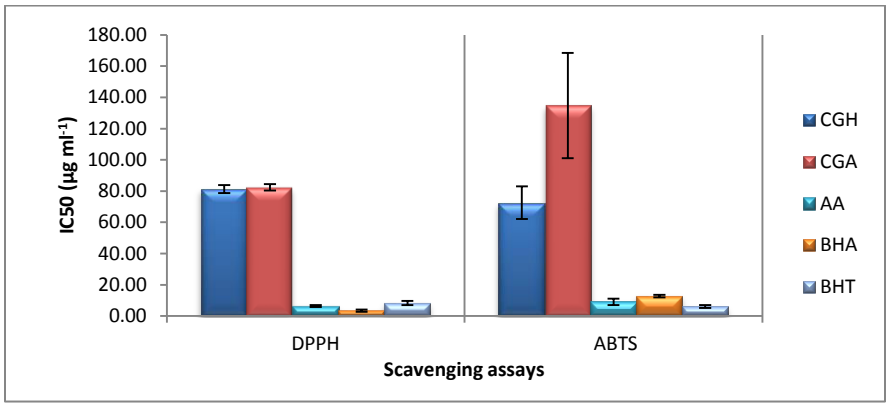


Fig 2: Comparison of IC_{50} of two free radical scavenging activity (DPPH and ABTS) of two different extracts of *C. griffithii* with standards.

Further, the antimicrobial activity of the extracts were largely comparable with different standards used did not produce any correlation pattern with the respective total phenol and total flavonoid content. Although, a linear relation with volatile oil, α-pinene, caryophyllene β-pinene, myrcene and limonene was observed, which are well known to impart antimicrobial value to plant species [36]. Antimicrobial activity of *C. griffithii* needles against various bacterial strains was largely in agreement with Moirangtham *et al*., 2012 [17], who performed similar test done using bark extract. The present investigation records *S. aureus* and *L. monocytogenes*, the most sensitive bacteria for organic solvent used.

The present work done on the phytochemical analysis, antioxidant activity and antimicrobial activity, together with previous investigation [17] (Moringtham *et al*., 2012), clearly suggests that *C. griffithii* bark as well as leaves are enrich parts of the plant, raveling various pharmacological effects, and could be potential source of natural antioxidant and antimicrobial agents. As this plant of very much useful ethanomedicinally and pharmacologically, but this plant species is less available and becoming endangered, requires conservation and propagation to save it in future.

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Conflicts of Interest

It is declared that there is no conflict of interest.

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