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Arindam Ghosh
Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Rajarammohanpur, Siliguri, Darjeeling, West Bengal, India

Sumedha Saha
Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Rajarammohanpur, Siliguri, Darjeeling, West Bengal, India

Soumya Majumder
Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Rajarammohanpur, Siliguri, Darjeeling, West Bengal, India

Sourav Chakraborty
1. Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Rajarammohanpur, Siliguri, Darjeeling, West Bengal, India
2. Post Graduate Department of Botany, Darjeeling Government College, Darjeeling, West Bengal, India

Malay Bhattacharya
Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Rajarammohanpur, Siliguri, Darjeeling, West Bengal, India

Corresponding Author:
Malay Bhattacharya
Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Rajarammohanpur, Siliguri, Darjeeling, West Bengal, India

In vitro assessment of the antioxidant and antibacterial activities of some shade tree barks from tea plantation of Terai region of West Bengal

Arindam Ghosh, Sumedha Saha, Soumya Majumder, Sourav Chakraborty and Malay Bhattacharya

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Abstract
The aim of this research was to evaluate the antioxidant and antibacterial activities of six shade tree barks. Barks of six plants were subjected to formulate the extracts with nine different nonpolar and polar solvents like hexane, chloroform, ethanol, methanol etc. The six bark extracts were assessed for their free radical scavenging and antibacterial potentialities by employing the DPPH scavenging assay and well diffusion methods, respectively. All the extracts were found to be more or less exhibited DPPH scavenging activity but methanolic extracts of *Senna siamea*; *Derris robusta*; *Leucaena leucocephala* were having the highest scavenging abilities. All the plants showed low to high antibacterial ability against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* where chloroform and aqueous extracts as well as all the extracts of *Melia azedarach* had no effect at all. So, this present study accomplish that the bark extracts of these shade trees could be exploited to develop the natural bioactive agents although further research are needed.

Keywords: Shade tree, bark extracts, antioxidant, antibacterial, *Escherichia coli*

Introduction
Nature is the source of the plethora of bioactive compounds where plants, herbs play a major role and large portion from these has been shown to possess therapeutic uses against the treatment of various pathologies like cancer, diabetes, neurodegenerative, cardiac diseases etc. [1]. Infectious disease caused by bacteria, fungi, protozoa etc. is the reason for the increasing number of mortality rate in developing countries and antibiotic resistant bacteria is the major factor behind this. So, presently, an increasing number of research are being going on to find out the natural bioactive compounds with having medicinal properties because of their worth in treating several diseases. Nearly 33% of drugs are being used throughout the globe are projected to be plant derivatives [2]. It has already been proven that many diseases are initiated by various xenobiotics including radiation, synthetic drugs, smoking, environmental agents, Reactive oxygen species (ROS) etc. and excessive ROS cannot be depleted or neutralized by endogenous pro-oxidants [2]. Traditionally, many plants have been used as medicinal plants because of their therapeutic uses against various diseases. In the tea plantation of terai region, many shade plants such as *Dalbergia sissoo*, *Derris robusta*, *Acacia lenticularis*, *Indigofera teysmanii*, etc. are used as to protect tea bushes but the bioactive properties of these all trees are not studied yet. *Dalbergia sissoo* acts a good antinociceptive, anthelmintic, anti-spermatogenic, osteogenic, anti-diarroheal etc. agent [3]. *Albizia odoratissima* has been found to be effective against leprosy, ulcers, cough etc. Shade trees like *Albizia procera* and *Albizia lebbeck* were found to have hepatoprotective, cardiotonic, lipid-lowering, hypoglycemic activity [4]. Ghosh *et al*. 2020 and Ghosh *et al*. 2021 [5, 6] has showed the antioxidant, antibacterial properties of leaves extracts of *Albizia odoratissima*, *Dalbergia sissoo*, *Derris robusta*, *Albizia lebbeck*, *Melia azedarach* but bioactive assets regarding their bark properties are yet to be discovered. So, aim of this study was to exploit the phytochemicals, antioxidant and antibacterial potent of six tea plantation shade trees of North Bengal.

Materials and Methods

Sample collection

Six shade trees *viz*. *Dalbergia sissoo* Roxb. (DS); *Derris robusta* (Roxb. ex DC.) Benth. (DR); *Senna siamea* (Lam.) Irwin et Barneby (SS); *Acacia lenticularis* Buch. -Ham. ex Benth. (AL); *Leucaena leucocephala* (Lam.) de Wit (LL) and *Melia azedarach* L. (MA) were chosen for this study and fresh barks from these selected plants were collected from the tea plantation of University of North Bengal (26º42′47.1″N, 88º20′54.2″E).

Sample preparation

Barks from these six shade trees were washed with tap water; surface was dried and ground to fine dust in the presence of liquid nitrogen [5]. 3g of each grounded samples were extracted in nine different nonpolar to polar solvents *viz*. Hexane(H), Benzene(B), Chloroform(C), Diethyl ether(D), Ethyl acetate(E), Ethanol (Et), Methanol(M), and Water(W) for 48 hours. The extracts were then filtered, stored in a refrigerator for further analysis.

Qualitative analysis

Qualitative analysis for the detection of bioactive compounds, i.e., tannin, coumarin, cardiac glycosides, steroid, flavonoids and terpenoids was done by following the protocol of Das *et al*. 2020 and Majumder *et al*. 2021 [7, 8].

Test for flavonoids

A few drops of 10% $FeCl_3$ solution were added to 1 ml of bark extracts. Formation of a green or blue colour indicated the presence of flavonoids.

Test for coumarin

A few drops of NaOH solution were added to 1 ml of bark extracts. Yellow coloration indicates the presence of coumarin.

Test for cardiac glycosides

0.5 ml of the sample was evaporated and dissolved in 1ml glacial acetic acid. 1 drop of 10% $FeCl_3$ solution followed by 1ml of concentrated H_2SO_4 was added by the side of the test tube. The appearance of a brown colour ring at the interface indicated the presence of cardiac glycosides.

Test for steroid

0.5 ml of leaf and bark extracts were evaporated and dissolved in 2 ml chloroform, and then 2 ml of concentrated H_2SO_4 was introduced carefully by the sidewall of the test tube. The formation of a red colour ring confirmed the presence of Steroids.

Test for tannin

To 0.5ml leaf and bark extracts were added with a few drops of HNO_3. The reddish to the yellow colour of the solution indicated the presence of tannins.

Test for terpenoids

To test the presence of terpenoid, 250 µl bark extract was evaporated, and the remaining was dissolved in chloroform, and concentrated H_2SO_4 was added in the test tubes. The formation of red to reddish-brown coloration at the base confirmed the presence of terpenoids.

Antioxidant assay {*Free radical (DPPH) scavenging assay*}

Free radical (2,2-diphenyl-1-picrylhydrazyl or DPPH) scavenging assay was performed by following the protocol of Ghosh *et al*. 2021 [6]. 200 µl of each bark extracts with a concentration of 500 mg/ml were added to 3 ml of 0.2 mM DPPH in methanol solvent. Then the mixture was vortexed for 30 minutes in the dark and kept at room temperature. The absorbance was measured in UV-Vis spectrophotometer at 517 nm. Ascorbic acid (µg/ml methanol) standard curve was taken as standard. DPPH scavenging activity was shown as a percentage of inhibition.

In vitro antibacterial assay

Antibacterial assay was done by following well diffusion method [9]. Two Gram-positive and two Gram-negative bacteria i.e., *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS), *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP) were considered for this study. Bark extracts of nine different solvents were dried out (500 mg/ml) and dissolved in 1000 µl of Dimethyl sulfoxide (DMSO). Mueller Hinton Agar was chosen for this experiment and 100µl of inoculum was given into each petri dish and the media was poured and semi-solidified. Nine wells were created with sterile cork borer for 9 different solvent extracts. Then, 100µl of each bark extracts were pipetted into the wells. After that, plates were nurtured at 37º C for 24 hours. Antibacterial susceptibility assay was determined by measuring the diameter of the inhibitory zone surrounding the well (0.8cm) containing the bark extracts and data was recorded.

Result and Discussions

Determination of preliminary phytochemical of different bark extracts

Preliminary phytochemicals estimation of those bark extracts for Flavonoid, Tannin, Coumarin, Terpenoid, Steroid, Cardiac glycosides are shown in Figure 1. Phytochemical groups like these are present in all the barks extracts with different solvents. Tannin being an astringent can be used as wound healing and antibacterial agent [5]. Tannin has only been found to be higher in the extracts of DS and little much in the acetone to methanolic extracts of MA. Cardiac glycosides and steroid have been found in all the extracts and comparatively found higher in the extracts of DS and SS. Cardiac glycosides can be used against congestive heart failure. Coumarin an anticoagulant agent used in the treatment of Pulmonary embolism (https://www.drugs.com/drug-class/coumarins-and-indandiones.html) has present in all the extracts except SS. Bioactive compound Terpenoid groups are found in every extract excluding DR. In case of flavonoid, none of the extracts have given positive results except in the aqueous extract of DS, as it was also previously reported that stem bark of this plant has great source of flavonoids [10]. Phytochemicals found in the bark extracts of these shade trees directs their potential for making of new remedies due to the existence of phytochemicals.

Fig 1: Heat map of different phytochemicals present in different bark extracts

Antioxidant activity of bark extracts (DPPH scavenging assay)

Antioxidant activity generally shows the capability of sample to scavenge the free radicals (DPPH). This experiment was supposed to be done as an indicator of antioxidant activity of samples. Different solvent extracts showed various results depending on their polarity. All the methanol extracts of bark, DR (91.72% or 194.36 µg AAE/G), SS (87.12% or 183.86 µg AAE/G), LL (78.77% or 164.79 µg AAE/G), AL (50.05% or 99.22 µg AAE/G) showed highest level of scavenging activity except the bark extracts of DS and MA. In DS (91.84% or 194.63 µg AAE/G) and MA (91.65% or 194.20 µg AAE/G) highest DPPH scavenging activity was recorded in benzene and acetone extracts. Minimum scavenging activity was found in all the hexane extract of bark except DR, where chloroform extract showed less activity (11.68% or 11.62 µg AAE/G) and no activity was found in the chloroform extract of AL. Results showed in Table 1 depicts that all the plants have a common preference towards polar solvents except DS. Methanol may be considered as a perfect solvent for the extraction of antioxidant molecules from barks of SS, DR, LL, AL, where for DS and MA the perfect solvents were benzene and acetone respectively. Stem bark of DS has an amusing number of flavonoids compounds [10] and Ghosh *et al*. 2020 [5], earlier also reported that the leaves of *Dalbergia sissoo*, *Derris robusta*, *Leucaena leucocephala*, *Melia azedarach*, *Acacia lenticularis* and *Senna siamea* have had good antioxidant activity. Ntalli & Caboni 2014 [11], have shown in their study that the leaf extract of *Melia azedarach* has antioxidant activity and can give protection from H_2O_2-induced cellular damage in lymphocytes. Kaur *et al*. 2006 [12], have found in their study that the flower extracts of *Senna siamea* have superoxide (O2•−), hydrogen peroxide (H_2O_2) and nitric oxide scavenging activity of 93.3%, 96.9% and 89.3% with a concentration of 50 µg/ml respectively. Quercetin-7-O-α-rhamnopyranosyl-(1”’→2”)-β-glucopyranoside a major compound of LL has great antioxidant activity, reported by Mohammed *et al*., 2015 [13]. So, these previous studies also proved the significance of having antioxidant compounds belonging to these trees.

Table 1: DPPH scavenging percentage and equivalent microgram Ascorbic acid /G scavenging activity of six shade tree bark extracts

	SS		DR		DS		LL		AL		MA	
	DPPH %	microG AAE/G	DPPH%	microG AAE/G	DPPH%	microG AAE/G	DPPH%	microG AAE/G	DPPH%	microG AAE/G	DPPH%	microG AAE/G
H	2.99	-8.22	14.59	18.26	26.57	45.61	8.83	5.11	8.31	3.92	9.46	6.55
B	27.39	47.49	17.56	25.04	91.84	194.63	21.48	33.99	19.52	29.52	29.28	51.80
C	31.22	56.23	11.68	11.62	80.96	169.79	14.55	18.17	0	-15.05	13.64	16.09
D	49.23	97.35	24.45	40.77	88.82	187.74	41	78.56	18.38	26.92	28.23	49.40
E	83.17	174.84	18.07	26.21	83.97	176.66	33.29	60.96	23.42	38.42	33.15	60.64
A	56.59	114.15	76.73	160.13	59.04	119.75	66.51	136.80	36.12	67.42	91.65	194.20
Et	67.49	139.04	69.85	144.43	61.61	125.61	61.84	126.14	41.21	79.04	90.86	192.39
M	87.12	183.86	91.72	194.36	70.13	145.07	78.77	164.79	50.05	99.22	84.9	178.79
W	11.65	11.55	13.06	14.77	15.95	21.37	12.19	12.78	6.89	0.68	9.49	6.62

*DPPH (% scavenging) and **Ascorbic acid equivalent (µg AE / ml) was calculated from the standard curve, y=0.876x+6.591, R^2 = 0.993

Antibacterial activity

The nine solvent extracts from different shade tree showed variable inhibition zones against two gram positive and gram-negative bacteria. The results of inhibition zones against bacteria have been shown in Table 2, where the nonpolar to polar solvents responded unevenly towards extraction of antibacterial compounds from the barks of shade tree and inhibition zones were produced against bacterial growth but the chloroform and aqueous extracts were found to be irrelevant. The highest summation of inhibition zones produced by different solvents were ethanol (6.75cm), ethyl acetate (6.15cm), acetone (5.8cm), methanol (4.8cm), diethyl ether (4.15cm), benzene (2.4cm) and hexane (1.1cm) respectively, but there was no summation of inhibition found in case of chloroform and water extract. The maximum summation of inhibition zones against four different organisms were mostly created by DS (10.85cm), followed by DR(9.1cm), LL(6.55cm), SS(3.05cm) and AL(1.6cm), but interestingly there was no inhibition zone created by any of the extract of MA. Summation of inhibition zone of bark extracts of DS showed best results against one-gram positive BS(6.6cm) and one-gram negative bacteria EC(4.25cm), followed by the extracts of DR which exhibited the growth of *Staphylococcus aureus* by summation of 9.1cm and extracts of LL inhibited the growth of SA, where summation of inhibition zones was 2.75cm. DS previously reported as a good antibacterial agent against *Staphylococcus aureus*, *Micrococcus leteus*, and *Enterobacter aerogenes* [14, 15], as this plant possess antimicrobial compound 1,2-benzenedicarbxylic acid dibutyl ester and 5-nitro-2,4 (1H,3H)-pyrimidine [16]. Paul *et al*. 2019 and Ghosh *et al*. 2020 [17, 5] previously showed in their study about the antimicrobial activity of DR. Antimicrobial compounds like 7-cinnamoyl toosendanin, 21b–methylmelianodiol, meliarachin D, meliarachin H of *M. azedarach* have been reported active against Micrococcus luteus ATCC 9341 and *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus, Bacillus subtilis* [11]. Another study on *Leucaena leucocephala* showed that the methanolic leaves extract have found to be effective against *E. coli* and *Bacillus subtilis* [18]. So, these previous reports also clarified the existence of antibacterial compounds in these trees.

Table 2: Inhibition zone created by the bark extracts of six shade trees

Solvents	Micro Organisms	DS	SS	DR	AL	MA	LL
Hexane	EC	——	——	——	——	——	——
	KP	——	——	——	——	——	——
	BS	——	——	——	——	——	——
	SA	——	——	1.1	——	——	——
Benzene	EC	——	——	——	——	——	——
	KP	——	——	——	——	——	——
	BS	1.1	——	——	——	——	——
	SA	——	——	1.3	——	——	——
Chloroform	EC	——	——	——	——	——	——
	KP	——	——	——	——	——	——
	BS	——	——	——	——	——	——
	SA	——	——	——	——	——	——
Diethyl ether	EC	1.1	——	——	——	——	——
	KP	——	——	——	——	——	——
	BS	1	——	——	——	——	——
	SA	——	——	1.2	——	——	0.85
Ethyl acetate	EC	1.1	0.95	——	——	——	——
	KP	——	——	——	——	——	0.9
	BS	1	——	——	——	——	——
	SA	——	——	1.3	——	——	0.9
Acetone	EC	1.05	——	——	——	——	——
	KP	——	——	——	——	——	0.95
	BS	1.2	——	——	——	——	——
	SA	——	——	1.6	——	——	1
Ethanol	EC	——	1.1	——	——	——	——
	KP	——	——	——	——	——	0.95
	BS	1	——	——	——	——	——
	SA	——	1	1.1	1.6	——	——
Methanol	EC	1	——	——	——	——	——
	KP	——	——	——	——	——	1
	BS	1.3	——	——	——	——	——
	SA	——	——	1.5	——	——	——
Water	EC	——	——	——	——	——	——
	KP	——	——	——	——	——	——
	BS	——	——	——	——	——	——
	SA	——	——	——	——	——	——

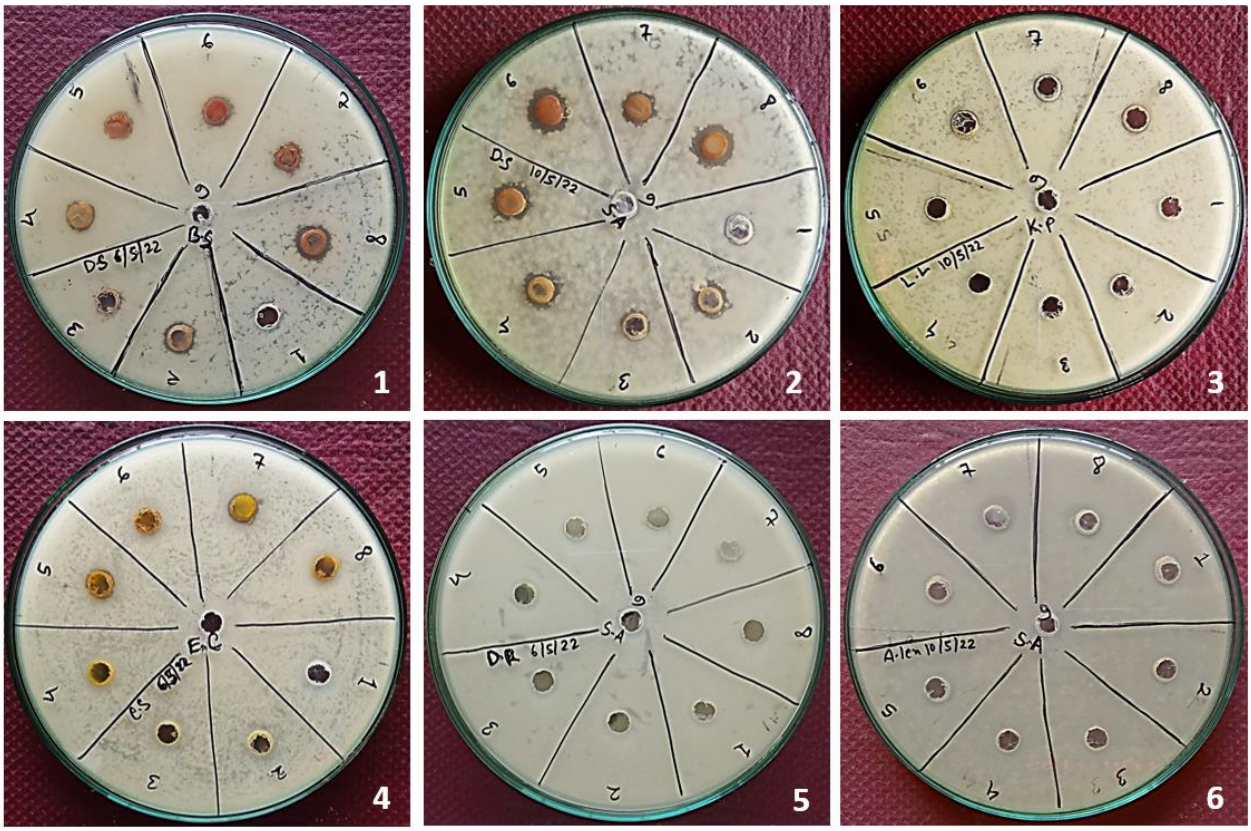

Fig 2: Representation of antibacterial assay, where 1-6 no plates indicating the susceptibility of different bacterial culture against bark extracts of DS, LL, SS, DR and AL

Conclusion

Tea, one of the most important agro-based industry which supports India's economy. So, shade trees planted in most of the tea gardens in India might also have some bioactive properties which may be explored. This present study conclude that shade trees are full of phytochemicals like terpenoids; steroids; cardiac glycosides; coumarin etc. High percentage of antioxidant as well as antibacterial properties were perceived in some of the shade tree barks, although they have solvent preferences. Solvents from benzene to methanol were found to be more potent for preliminary phytochemical study. Methanol was found to be significant solvents for extraction of free radical scavenging molecules in case of SS; DR; LL and AL, whereas benzene and acetone was proved to be good for DS and MA. In antibacterial assay, from ethyl acetate to methanol solvents were proven to be excellent for the extraction of antibacterial compounds. Therefore, it can be concluded that selection of proper solvent is necessary for *in vitro* studies. So, this research study highlights that these shade trees could be an excellent source of bioactive agents, which could further promote future research to exploit these trees in an effective way.

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