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Appendix – A

List of Publication

RESEARCH ARTICLE

- Bose, D., Sarkar, I., **Labar, R**., Oshone, R., Ghazal, S., Morris, K., ... & Sen, A. (**2016**). Comparative genomics of Prauserella sp. Am3, an actinobacterium isolated from root nodules of Alnus nepalensis in India. ***Symbiosis***, *70*(1), 49-58.

- **Labar, R.,** Sen, A., & Bhattacharya, M. (**20**). Effect of solvents on qualitative and quantitative phytochemical constituent profiles of fresh leaves of TV26. ***NBU J Plant Sc***. *Vol*, *11*, 115-123.

- **Labar, R.,** Sarkar, I., Sen, A., & Bhattacharya, M. (**2019**). Effect of solvent with varying polarities on phytochemical extraction from mature tea leaves and its evaluation using biochemical, antimicrobial and in-silico approaches. ***Int Res J Pharm***, *10*(8), 59-67.
- **Labar, R.,** Kar, P., Biswas, P., Sen, A., & Bhattacharya, M. (**2021**). Evolution of matK Gene among the Elite Tea Clones (*Camellia sinensis*) Revealed by Nucleotide Substitution within the Consensus Region**.** ***J Appl Biol Biotechnol,*** *Vol*, *9*(1), 32-40.

REVIEW ARTICLE

- **Labar, R.,** and Sen, A. (**2015**). Efficacy of Anthocyanin in Production of Remedial Tea. *NBU J Plant Sc*. *Vol*, *9*(1), 18-32.

AWARDS AND HONOUR

- Outstanding paper award: 3rd Regional Science Congress

- Outstanding paper award: 27th West Bengal state Science Congress

Appendix – B

Buffers and chemicals used for DNA fingerprinting studies

CTAB- buffer

100mM Trizma Base (Sigma, Cat# T1503) (pH-8.0)

20mM EDTA (Merck India, Cat# 60841801001730) (pH-8.0)

1.4 M NaCl (Merck India, Cat#60640405001730)

2% (w/v) CTAB (Hexadecyl cetyl trimethyl ammonium bromide) (Sigma, Cat# H6269)

12.11g of molecular grade Trizma base was dissolved in 400 ml double distilled water, pH was adjusted to 8.0 and was divided into two parts of equal volume. To one part 7.44g EDTA was added and to the other part 81.8g NaCl and 20g CTAB. Both the parts were than mixed and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121°C and 15 psi for 20 mins and stored at room temperature for further use.

Note: Add 1% PVP (Polyvinylpyrrolidone) (Sigma, Cat #P5288) and 0.3% β-mercaptoethanol (Sigma, Cat# M3148) just before use.

CTAB/NaCl

CTAB (Sigma, Cat# H6269) =10gm

NaCl (Merck India, Cat#60640405001730) = 4.1gm

Final volume=100ml

In 80ml double distilled water, 4.1gm of NaCl was dissolved. Slowly 10gm of CTAB was added with continuous heating and stirring until the solution becomes clear. The final volume

of the solution was adjusted to 100ml by adding ddH2O. The solution was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

5X TBE (Tris-borate-EDTA) buffer

Trizma base (Sigma, Cat# T1503) = 27 gm

Boric acid (Sigma, Cat# 15663) = 13.75 gm

0.5M EDTA (pH 8.0) =1.86 gm

All the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

1X TE

Tris- Cl (pH 8.0) (i.e., 10Mm) =0.6055gm

EDTA (pH 8.0) (i.e., 1mM) =0.186 gm

Both the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

3M Sodium Acetate (Sigma, Cat# S9513)

The required amount of sodium acetate i.e.,12.31 g was dissolved in 50ml double distilled water prior to autoclaving. The solution was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

6X gel loading buffer

TYPE 3:

0.25% Bromophenol blue (Sigma, Cat# B0126)

0.25% Xylene cyanol FF (Sigma, Cat# X4126)

30% Glycerol (Merck India, Cat#61756005001730) in water

Store at 4ºC.

RNase A

The RNase A enzyme (Sigma, Cat# R4875) was dissolved at a concentration of 10mg/ml in

0.01M sodium acetate (Sigma, Cat# S9513) (pH 5.2). The solution was heated at 100ºC for 15 minutes in a water bath and allowed to cool slowly to room temperature. The pH was adjusted by adding 1/10 volume of 1M Tris- Cl (pH 7.4) and stored at -20ºC for further use.

Note: Both 0.01M sodium acetate and 1M Tris-Cl were prepared and autoclaved at 121ºC

and 15 psi for 20 mins prior to use.

SDS (10%)

SDS (Sigma, USA. Cat #L4390) =5gm

Final volume=50ml

In 20ml of ddH2O, 5gm of SDS was added and heated to dissolve. The final volume was made upto 50ml and autoclaved for future use.

Appendix – C

SEQUENCE SUBMITTED IN NCBI

1. MH649284 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Ambari Vallai 2 maturase K (matK) gene, partial cds; chloroplast.
2. MH791417 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Happy Valley 39 maturase K (matK) gene, partial cds; chloroplast
3. MH920315 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Takdah 253 maturase K (matK) gene, partial cds; chloroplast.
4. MH920316 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Nandadevi maturase K (matK) gene, partial cds; chloroplast.
5. MH920317 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone MB - 6 maturase K (matK) gene, partial cds; chloroplast.
6. MH920318 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Teesta Valley 1 maturase K (matK) gene, partial cds; chloroplast.
7. MH920319 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Kopati 1/1 maturase K (matK) gene, partial cds; chloroplast.
8. MH393392- **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Badamtam 15/263 maturase K (matK) gene, partial cds; chloroplast.

9. MH393393 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Balasun 7/1A/76 maturase K (matK) gene, partial cds; chloroplast.
10. MH393394 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Phoobsering 312 maturase K (matK) gene, partial cds; chloroplast.
11. MK393395 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Bannockburn 777 maturase K (matK) gene, partial cds; chloroplast.
12. MK393396 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Sundaram maturase K (matK) gene, partial cds; chloroplast.
13. MK393397- Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Takdah 135 maturase K (matK) gene, partial cds; chloroplast.
14. MK393398 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Bannockburn 688 (matK) gene, partial cds; chloroplast.
15. MK393399 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Golconda maturase K (matK) gene, partial cds; chloroplast.
16. MK393400 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Rungli Rungiliot 17/144 maturase K (matK) gene, partial cds; chloroplast.
17. MK393401 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Balasun 9/3/76 maturase K (matK) gene, partial cds; chloroplast.
18. MK393402- Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Chiradew Parbat1 maturase K (matK) gene, partial cds; chloroplast.
19. MK393403 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Phoobsering 1404 maturase K (matK) gene, partial cds; chloroplast.

20. MK393404 - Kar, P., **Labar, R.,** Bhattacharya, M., Biswas, P. and Sen, A. *Camellia sinensis* clone Phoobsering 1258 maturase K (matK) gene, partial cds; chloroplast.
21. MK393405 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Rungli Rungiliot 4/5 maturase K (matK) gene, partial cds; chloroplast.
22. MK424865 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Sikkim 1 maturase K (matK) gene, partial cds; chloroplast.
23. MK424866 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Takdah 145 maturase K (matK) gene, partial cds; chloroplast.
24. MK424867 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Takdah 246 K (matK) gene, partial cds; chloroplast.
25. MK424868 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Tocklai Variety 19 maturase K (matK) gene, partial cds; chloroplast.
26. MK424869 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Tocklai Variety 14 maturase K (matK) gene, partial cds; chloroplast.
27. MK424870 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Takdah 78 maturase K (matK) gene, partial cds; chloroplast.
28. MK424871 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Bannockburn 157 maturase K (matK) gene, partial cds; chloroplast.
29. MK424872 - Bhattacharya, M., **Labar, R.,** Kar, P., Biswas, P. and Sen, A. *Camellia sinensis* clone Takdah 383 maturase K (matK) gene, partial cds; chloroplast.
30. MN480321 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Thurbo 3 maturase K (matK) gene, partial cds; chloroplast.
31. MN480322 - Labar**, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Thurbo 9 maturase K (matK) gene, partial cds; chloroplast.

32. MZ130930 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Thurbo 3 ribosomal protein S16 (rps16) gene, partial cds; chloroplast.
33. MZ130931 -- **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone Thurbo 9 ribosomal protein S16 (rps16) gene, partial cds; chloroplast.
34. MZ130932 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone BS 7/1A/76 ribosomal protein S16 (rps16) gene, partial cds; chloroplast.
35. MZ130933 - **Labar, R.,** Kar, P., Biswas, P., Bhattacharya, M. and Sen, A. 2019. *Camellia sinensis* clone clone B-777 ribosomal protein S16 (rps16) gene, partial cds; chloroplast.

Appendix - D

Table 1: Phytoconstituents of tea extract (Assam) characterized by GC-MS

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	4.683	941660	0.08	2,4-Dimethyl-1-heptene
2	4.740	847411	0.08	2-HYDROXY-2-METHYL-4-PENTANONE (DIACETONE
3	5.086	1310017	0.12	4,4-Dimethoxy-2-methyl-2-butanol
4	5.451	13969189	1.26	BUTANOIC ACID, 3-METHYL-
5	5.528	14008026	1.26	1-BUTANOL, 3-METHYL-, ACETATE
6	6.346	7918761	0.71	HYDROPEROXIDE, 1,1-DIMETHYLETHYL
7	7.490	1467464	0.13	BUTANE, 1,1-DIETHOXY-3-METHYL-
8	8.043	2803378	0.25	PENTANE, 1-(1-ETHOXYETHOXY)-
9	8.253	1463283	0.13	ETHANE, PENTACHLORO-
10	8.364	5782140	0.52	TETRAHYDRO-2-(TETRAHYDRO-2-FURFURYL)-FURA
11	9.064	3123505	0.28	ISOBUTYLISOVALERATE
12	9.224	8266587	0.75	Propanoic acid, 2-methyl-, 3-methylbutyl ester
13	9.318	1191227	0.11	PROPANOIC ACID, 2-METHYL-, 3-METHYLBUTYL ES
14	9.835	1393664	0.13	TETRAHYDRO-2-(TETRAHYDRO-2-FURFURYL)-FURA
15	10.232	3538133	0.32	BUTANOIC ACID, 3-METHYL-, BUTYL ESTER
16	10.511	4426347	0.40	ISO AMYL N-BUTYRATE
17	11.044	7472070	0.67	Ethane, hexachloro-
18	11.177	5464583	0.49	1,3-Dioxan-4-one, 2-heptyl-6-methyl
19	11.699	3961589	0.36	2-Furanol, tetrahydro-2,3-dimethyl-, trans-
20	11.828	48697428	4.39	ISOPENTYL 2-METHYLBUTANOATE
21	11.919	7053237	0.64	BUTANOIC ACID, 2-METHYL-, 2-METHYLBUTYL EST
22	12.081	158324513	14.27	PENTANOIC ACID, PENTYL ESTER
23	12.121	19795080	1.78	PENTYL 3-METHYLBUTANOATE
24	14.169	29317951	2.64	2-Butanol, 3-(2,2-dimethylpropoxy)-
25	14.305	14439683	1.30	NAPHTHALENE
26	14.406	1156236	0.10	PROPANOIC ACID, 2-METHYL-, 1-METHYLBUTYL ES
27	14.534	1283502	0.12	1-DODECANOL
28	14.900	19681178	1.77	PROPANOIC ACID, 2-METHYL-, 3-METHYLBUTYL ES
29	15.279	2070734	0.19	2,4-OXAZOLIDINEDIONE, 5,5-DIMETHYL-
30	15.422	9573134	0.86	Butane, 2,2'-[methylenebis(oxy)]bis[2-methyl-
31	16.463	6206086	0.56	Pentan-2-yl 2-methylbutanoate
32	16.924	12677893	1.14	TERT-PENTYL BUTYRATE #
33	17.108	75090507	6.77	PENTANE, 1,1'-OXYBIS-
34	17.234	1854308	0.17	TERT-PENTYL BUTYRATE #
35	17.609	4045864	0.36	Oxalic acid, 2-ethylhexyl pentyl ester
36	17.832	1932328	0.17	Sulfurous acid, octadecyl 2-propyl ester
37	18.057	1586207	0.14	2-Isopropyl-5-methyl-1-heptanol
38	18.851	2168485	0.20	Butanoic acid, 2,2-dimethylpropyl ester
39	19.988	2690410	0.24	2-METHYLPROPIONYL PHENYLSELENO SULFIDE
40	20.100	45543516	4.11	diethyl 2-hydroxy-3-(tetrahydrofuran-2-yl)succinate
41	20.204	77426697	6.98	Tripentyl orthoformate
42	20.279	1435480	0.13	TETRADECANE
43	20.813	4148818	0.37	Propanoic acid, 2-methyl-, pentyl ester

Peak#	R.Time	Area	Area%	Name
44	22.903	6216186	0.56	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-
45	23.595	1908744	0.17	Eicosane
46	24.993	3799147	0.34	1-HEXADECENE
47	25.174	2182662	0.20	Hexadecane
48	28.380	1314281	0.12	Eicosane
49	28.674	1972073	0.18	OCTADECANOIC ACID
50	29.442	3860426	0.35	1-OCTADECENE
51	29.593	1621985	0.15	EICOSANE
52	30.324	5566676	0.50	Caffeine
53	31.647	1983574	0.18	7,9-DITERT-BUTYL-1-OXASPIRO[4.5]DECA-6,9-DIENE-
54	32.107	1222258	0.11	HEXADECANOIC ACID, METHYL ESTER
55	32.634	1579085	0.14	Dibutyl phthalate
56	32.827	10709536	0.97	HEXADECANOIC ACID
57	33.475	3716006	0.33	n-Tetracosanol-1
58	33.605	1813258	0.16	EICOSANE
59	34.422	1686006	0.15	Hexadecanoic acid, trimethylsilyl ester
60	35.476	4245849	0.38	Heneicosane
61	36.573	2258057	0.20	Octadecanoic acid
62	37.254	2544252	0.23	n-Tetracosanol-1
63	37.373	15575865	1.40	DOCOSANE
64	38.066	1292782	0.12	3-METHYLBUTYL DECANOATE
65	38.840	27213621	2.45	DOCOSANE
66	39.913	35284249	3.18	PENTACOSANE
67	40.776	30259090	2.73	PENTACOSANE
68	41.013	1230102	0.11	OCTADECANAL
69	41.254	2688734	0.24	Tetratetracontane
70	41.551	32340484	2.92	TETRACONTANE
71	42.054	4042684	0.36	Tetratetracontane
72	42.139	2857543	0.26	Octadecane, 9-ethyl-9-heptyl-
73	42.380	34998234	3.16	TETRACONTANE
74	42.890	1511823	0.14	TRICOSANE
75	42.947	5818265	0.52	Tetratetracontane
76	43.049	6031542	0.54	Hexacosane, 9-octyl-
77	43.241	2605687	0.23	1-Cyclopentyleicosane
78	43.323	36774495	3.32	TETRACONTANE
79	43.462	4625992	0.42	Squalene
80	43.691	1302174	0.12	2-Cyclohexylnonadecane
81	43.825	1489030	0.13	CELIDONIOL, DEOXY-
82	43.916	2976271	0.27	Tetratetracontane
83	43.981	4512312	0.41	Tetratetracontane
84	44.104	7465125	0.67	11,15-Dimethylpentatriacontane
85	44.422	45056493	4.06	TETRACONTANE
86	44.529	3354502	0.30	n-Heptadecylcyclohexane
87	44.817	1569506	0.14	Z-14-Nonacosane

Peak#	R.Time	Area	Area%	Name
88	44.890	1705818	0.15	CYCLOHEXANE, EICOSYL-
89	44.959	1585120	0.14	TRITETRACONTANE
90	45.137	2752709	0.25	TRICOSANE
91	45.209	2842808	0.26	Tetratriacontane
92	45.359	3311273	0.30	13,17,21-Trimethylheptatriacontane
93	45.735	29429447	2.65	TETRACONTANE
94	45.913	7238643	0.65	n-Heptadecylcyclohexane
95	46.250	4077668	0.37	1-Triacontanol
96	46.337	9403903	0.85	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9
97	46.571	4837342	0.44	NOROLEAN-12-ENE
98	47.337	14507781	1.31	PENTATRIACONTANE
99	47.516	1645312	0.15	a-Neooleana-3,12-diene
100	47.596	2164856	0.20	n-Heptadecylcyclohexane
101	48.010	5579470	0.50	Urs-12-ene
102	49.316	7571589	0.68	PENTATRIACONTANE
		1109278714	100.00	

Table 2 Phytoconstituents of tea extract (China) characterized by GC-MS.

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	4.737	951562	0.08	2-HYDROXY-2-METHYL-4-PENTANONE (DIACETONE
2	5.081	1407061	0.12	4,4-Dimethoxy-2-methyl-2-butanol
3	5.457	11929558	1.01	BUTANOIC ACID, 3-METHYL-
4	5.525	12949100	1.09	1-BUTANOL, 3-METHYL-, ACETATE
5	6.345	7891633	0.67	HYDROPEROXIDE, 1,1-DIMETHYLETHYL
6	7.490	1644077	0.14	BUTANE, 1,1-DIETHOXY-3-METHYL-
7	8.041	2829773	0.24	PENTANE, 1-(1-ETHOXYETHOXY)-
8	8.256	1794026	0.15	ETHANE, PENTACHLORO-
9	8.361	4581997	0.39	TETRAHYDRO-2-(TETRAHYDRO-2-FURFURYL)-FURA
10	9.059	2711807	0.23	ISOBUTYLISOVALERATE
11	9.220	7808394	0.66	Propanoic acid, 2-methyl-, 3-methylbutyl ester
12	9.313	1183931	0.10	PROPANOIC ACID, 2-METHYL-, 3-METHYLBUTYL ES
13	9.831	1130599	0.10	TETRAHYDRO-2-(TETRAHYDRO-2-FURFURYL)-FURA
14	10.229	3503651	0.30	BUTANOIC ACID, 3-METHYL-, BUTYL ESTER
15	10.506	4385203	0.37	ISO AMYL N-BUTYRATE
16	11.012	8973287	0.76	4,5-Octanediol, 2,7-dimethyl-
17	11.172	6044376	0.51	1,3-Dioxan-4-one, 2-heptyl-6-methyl
18	11.691	4173155	0.35	2-Furanol, tetrahydro-2,3-dimethyl-, trans-
19	11.826	48749178	4.12	ISOPENTYL 2-METHYLBUTANOATE
20	11.917	7123689	0.60	BUTANOIC ACID, 2-METHYL-, 2-METHYLBUTYL EST
21	12.076	160496607	13.55	PENTANOIC ACID, PENTYL ESTER
22	12.115	18650969	1.57	PENTYL 3-METHYLBUTANOATE
23	12.447	975139	0.08	2H-PYRAN, TETRAHYDRO-2-[(TETRAHYDRO-2-FURA
24	14.167	30003187	2.53	2-Butanol, 3-(2,2-dimethylpropoxy)-
25	14.299	8631565	0.73	NAPHTHALENE
26	14.403	1324213	0.11	PROPANOIC ACID, 2-METHYL-, 1-METHYLBUTYL ES
27	14.898	20052190	1.69	PROPANOIC ACID, 2-METHYL-, 3-METHYLBUTYL ES
28	15.276	2049766	0.17	2,4-OXAZOLIDINEDIONE, 5,5-DIMETHYL-
29	15.421	9262473	0.78	Butane, 2,2'-[methylenebis(oxy)]bis[2-methyl-
30	16.464	6127497	0.52	Pentan-2-yl 2-methylbutanoate
31	16.924	14366096	1.21	TERT-PENTYL BUTYRATE #
32	17.113	84538200	7.14	PENTANE, 1,1'-OXYBIS-
33	17.234	2146469	0.18	TERT-PENTYL BUTYRATE #
34	17.612	3790373	0.32	Oxalic acid, neopentyl octyl ester
35	17.829	1719951	0.15	2-Hexyldecyl acetate
36	17.884	1396155	0.12	Butanoic acid, anhydride
37	18.055	1285506	0.11	2-Isopropyl-5-methyl-1-heptanol
38	18.849	2187833	0.18	Butyric acid, 1-propylpentyl ester
39	19.087	1050635	0.09	Propanoic acid, 2-methyl-, 1,2,3-propanetriyl ester
40	19.315	1745230	0.15	1,3-Dioxane, 5-ethyl-2,2-dimethyl-
41	19.987	2808445	0.24	2-METHYLPROPIONYL PHENYLSELENO SULFIDE
42	20.098	45528177	3.84	diethyl 2-hydroxy-3-(tetrahydrofuran-2-yl)succinate
43	20.203	79417711	6.70	Tripentyl orthoformate

Peak#	R.Time	Area	Area%	Name
44	20.276	1725891	0.15	TETRADECANE
45	20.649	1618868	0.14	2-Butenoic acid, 1-methylethyl ester
46	20.811	4663100	0.39	Propanoic acid, 2-methyl-, pentyl ester
47	22.902	6012571	0.51	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-
48	23.594	1837488	0.16	Eicosane
49	24.992	3680355	0.31	1-HEXADECENE
50	25.174	2449935	0.21	Hexadecane
51	28.672	2018213	0.17	OCTADECANOIC ACID
52	29.440	3768722	0.32	1-OCTADECENE
53	29.593	1439797	0.12	EICOSANE
54	30.381	25396950	2.14	Caffeine
55	31.646	2027066	0.17	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
56	32.632	2488888	0.21	Dibutyl phthalate
57	32.830	12529090	1.06	HEXADECANOIC ACID
58	33.473	3524058	0.30	1-OCTADECENE
59	34.421	1706418	0.14	Hexadecanoic acid, trimethylsilyl ester
60	35.473	3728859	0.31	Heneicosane
61	36.574	7045354	0.59	Octadecanoic acid
62	37.252	2483297	0.21	n-Tetracosanol-1
63	37.369	14612691	1.23	DOCOSANE
64	38.838	26715832	2.26	DOCOSANE
65	39.912	36944873	3.12	PENTACOSANE
66	40.777	33438898	2.82	PENTACOSANE
67	41.253	2765827	0.23	Tetratetracontane
68	41.552	36692995	3.10	TETRACONTANE
69	42.053	4499247	0.38	Tetratetracontane
70	42.140	3081651	0.26	Octadecane, 9-ethyl-9-heptyl-
71	42.381	40946772	3.46	TETRACONTANE
72	42.890	1646893	0.14	TRICOSANE
73	42.947	6857133	0.58	Tetratetracontane
74	43.048	6760985	0.57	Hexacosane, 9-octyl-
75	43.240	2835215	0.24	1-Cyclopentyleicosane
76	43.323	43898109	3.71	TETRACONTANE
77	43.689	1318998	0.11	CYCLOHEXANE, EICOSYL-
78	43.825	1591084	0.13	CELIDONIOL, DEOXY-
79	43.916	4923851	0.42	Tetratetracontane
80	43.977	7288682	0.62	Tetratetracontane
81	44.103	8358966	0.71	TETRACONTANE
82	44.422	49583281	4.19	TETRACONTANE
83	44.528	3591126	0.30	n-Heptadecylcyclohexane
84	44.817	1776119	0.15	1-EICOSANOL
85	44.885	2053745	0.17	CYCLOHEXANE, EICOSYL-
86	44.956	1972063	0.17	TRITETRACONTANE
87	45.132	3391172	0.29	TRICOSANE

Peak#	R.Time	Area	Area%	Name
88	45.206	3208297	0.27	Hexatriacontane
89	45.355	3915551	0.33	13,17,21-Trimethylheptatriacontane
90	45.732	44696244	3.77	Docosanoic acid, docosyl ester
91	45.911	7634386	0.64	n-Heptadecylcyclohexane
92	46.252	3125807	0.26	1-EICOSANOL
93	46.688	4739472	0.40	Hexatriacontane
94	47.331	20061001	1.69	PENTATRIACONTANE
95	47.595	5853300	0.49	n-Heptadecylcyclohexane
96	48.001	3543885	0.30	Heptacosane, 1-chloro-
97	49.313	8767795	0.74	PENTATRIACONTANE
		1184557310	100.00	

Table 3: Phytoconstituents of tea extract (AV2) characterized by GC-MS

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	4.305	279010	0.52	Heptane, 2,4-dimethyl-
2	4.711	15208680	28.58	2-HYDROXY-2-METHYL-4-PENTANONE (DIACETONE
3	5.131	75414	0.14	OCTANE, 4-METHYL-
4	6.403	2431765	4.57	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
5	6.554	248137	0.47	BICYCLO[3.1.1]HEPT-2-ENE, 2,6,6-TRIMETHYL-
6	7.125	63520	0.12	NONANE, 2-METHYL-
7	7.313	273593	0.51	BICYCLO[3.1.0]HEXANE, 4-METHYLENE-1-(1-METHY
8	7.414	63413	0.12	BICYCLO[3.1.1]HEPTANE, 6,6-DIMETHYL-2-METHYLE
9	7.941	90963	0.17	1,3-CYCLOHEXADIENE, 2-METHYL-5-(1-METHYLETH
10	7.993	64009	0.12	BICYCLO[4.1.0]HEPT-3-ENE, 3,7,7-TRIMETHYL-
11	8.033	85066	0.16	Undecane, 5,6-dimethyl-
12	8.133	69327	0.13	HEXANE, 2,2,3,3-TETRAMETHYL-
13	8.199	213758	0.40	Octane, 3,3-dimethyl-
14	8.281	47689	0.09	BENZENE, 1-METHYL-4-(1-METHYLETHYL)-
15	8.369	191218	0.36	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETHENYL)-
16	8.423	143138	0.27	2-OXABICYCLO[2.2.2]OCTANE, 1,3,3-TRIMETHYL-
17	8.786	570544	1.07	Decane, 3,7-dimethyl-
18	8.885	281466	0.53	Decane, 3,7-dimethyl-
19	9.563	186001	0.35	Decane, 3,7-dimethyl-
20	9.657	146365	0.28	Hexane, 3,3,4-trimethyl-
21	10.618	62843	0.12	Decane, 1-iodo-
22	10.774	54187	0.10	NONANE, 3,7-DIMETHYL-
23	11.196	112822	0.21	HEPTADECANE
24	11.326	34735	0.07	Heptane, 3,4,5-trimethyl-
25	11.377	105264	0.20	Undecane, 2,6-dimethyl-
26	11.510	104600	0.20	Dodecane, 4-methyl-
27	11.795	91320	0.17	Sulfurous acid, 2-ethylhexyl isohexyl ester
28	11.880	55358	0.10	NONANE, 3,7-DIMETHYL-
29	11.946	561494	1.06	Benzene, 1,3-bis(1,1-dimethylethyl)-
30	12.112	191592	0.36	DECANE, 2,3,7-TRIMETHYL-
31	12.300	574484	1.08	Nonane, 5-(2-methylpropyl)-
32	12.424	86957	0.16	Undecane, 3,8-dimethyl-
33	12.510	58713	0.11	Undecane, 3,8-dimethyl-
34	12.618	68803	0.13	Decane, 3,7-dimethyl-
35	12.760	56353	0.11	Hexane, 3,3-dimethyl-
36	12.967	180836	0.34	Decane, 3,7-dimethyl-
37	13.559	47673	0.09	Decane, 1-iodo-
38	14.153	29629	0.06	3-OXO-1-CYCLOHEXENYL 2-METHYLPROPIONAT
39	14.196	46705	0.09	Decane, 2,5,6-trimethyl-
40	14.256	82061	0.15	Sulfurous acid, 2-ethylhexyl isohexyl ester
41	14.325	51059	0.10	Hexane, 3,3,4-trimethyl-
42	14.703	58862	0.11	PENTADECANE
43	14.779	78123	0.15	Sulfurous acid, 2-ethylhexyl isohexyl ester

Peak#	R.Time	Area	Area%	Name
44	14.837	43386	0.08	HEPTANE, 3,3,5-TRIMETHYL-
45	14.867	54626	0.10	Undecane, 3,4-dimethyl-
46	14.953	40840	0.08	Undecane, 3,8-dimethyl-
47	15.136	117326	0.22	Nonane, 5-(2-methylpropyl)-
48	15.193	546331	1.03	Heptadecane
49	15.280	44950	0.08	Nonane, 2,5-dimethyl-
50	15.315	32980	0.06	Nonane, 5-methyl-5-propyl-
51	15.505	648502	1.22	Phenol, 3,5-bis(1,1-dimethylethyl)-
52	15.624	41840	0.08	Nonane, 5-(2-methylpropyl)-
53	15.759	200674	0.38	Nonane, 5-butyl-
54	15.885	42477	0.08	Decane, 3,7-dimethyl-
55	16.480	55612	0.10	Diethyl Phthalate
56	16.653	71483	0.13	Octane, 2-methyl-
57	16.877	80747	0.15	Heptadecane, 2,6,10,15-tetramethyl-
58	17.113	50039	0.09	Octane, 3,5-dimethyl-
59	17.230	39182	0.07	1-PIPERAZINEETHANAMINE, 4-METHYL-
60	17.333	21828	0.04	Undecane, 3,8-dimethyl-
61	17.659	128321	0.24	Dodecane, 2-methyl-
62	17.716	388524	0.73	OCTADECANE
63	17.765	45848	0.09	Nonane, 5-(2-methylpropyl)-
64	17.820	80651	0.15	Nonane, 5-methyl-5-propyl-
65	18.031	117184	0.22	Dodecyl nonyl ether
66	18.153	33571	0.06	1-METHYL-1,2,4-TRIAZOLE-4-15N
67	18.203	90801	0.17	Nonane, 5-methyl-5-propyl-
68	18.257	29316	0.06	NONANE, 4,5-DIMETHYL-
69	19.205	1399143	2.63	Neophytadiene
70	19.477	18352952	34.49	Caffeine
71	19.655	460801	0.87	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
72	19.805	50137	0.09	Nonadecane
73	19.888	67777	0.13	2-Methyltetracosane
74	19.968	211675	0.40	Eicosane
75	20.335	39057	0.07	Nonane, 5-(1-methylpropyl)-
76	20.395	71431	0.13	Nonane, 5-methyl-5-propyl-
77	21.294	43598	0.08	OCTADECANE
78	21.660	24894	0.05	HEXADECANE
79	21.701	28941	0.05	Octane, 2-methyl-
80	21.934	200468	0.38	Phytol
81	21.998	166408	0.31	2-Methylhexacosane
82	22.385	75117	0.14	Dodecane, 2-methyl-
83	23.929	99635	0.19	2-methyloctacosane
84	28.753	628222	1.18	Squalene
85	29.727	326299	0.61	2-methyloctacosane
86	32.693	410095	0.77	2-methyloctacosane
87	33.255	1962515	3.69	Vitamin E

Peak#	R.Time	Area	Area%	Name
88	37.354	780592	1.47	STIGMASTA-7,22-DIEN-3-OL, (3.BETA.,5.ALPHA.,22E,2
89	38.532	1230945	2.31	24-Noroleana-3,12-diene
		53205290	100.00	

Table 4. Phytoconstituents of tea extract (TS569) characterized by GC-MS.

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	4.305	200603	0.45	Heptane, 2,4-dimethyl-
2	4.718	17134202	38.51	2-HYDROXY-2-METHYL-4-PENTANONE (DIACETONE
3	6.405	1591110	3.58	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
4	6.553	148471	0.33	BICYCLO[3.1.1]HEPT-2-ENE, 2,6,6-TRIMETHYL-
5	7.316	188820	0.42	BICYCLO[3.1.0]HEXANE, 4-METHYLENE-1-(1-METHY
6	7.994	65491	0.15	BICYCLO[4.1.0]HEPT-3-ENE, 3,7,7-TRIMETHYL-
7	8.034	78393	0.18	UNDECANE, 5,6-DIMETHYL-
8	8.135	57915	0.13	OCTANE, 2,4,6-TRIMETHYL-
9	8.201	206359	0.46	Octane, 3,3-dimethyl-
10	8.370	156799	0.35	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETHENYL)-
11	8.788	548475	1.23	Decane, 3,7-dimethyl-
12	8.886	269234	0.61	Decane, 3,7-dimethyl-
13	9.565	187744	0.42	Decane, 3,7-dimethyl-
14	9.660	129537	0.29	Hexane, 3,3-dimethyl-
15	11.197	126743	0.28	PENTADECANE
16	11.379	116268	0.26	Octane, 3,5-dimethyl-
17	11.512	113475	0.26	Dodecane, 4-methyl-
18	11.797	102577	0.23	PENTADECANE
19	11.948	556730	1.25	Benzene, 1,3-bis(1,1-dimethylethyl)-
20	12.113	208916	0.47	NONADECANE
21	12.302	616648	1.39	Nonane, 5-butyl-
22	12.428	93012	0.21	Nonane, 5-methyl-5-propyl-
23	12.512	64515	0.15	3-Ethyl-3-methylheptane
24	12.619	77773	0.17	Nonane, 5-(2-methylpropyl)-
25	12.967	199358	0.45	Decane, 3,7-dimethyl-
26	14.257	84577	0.19	Sulfurous acid, 2-ethylhexyl isohexyl ester
27	14.781	82594	0.19	Sulfurous acid, 2-ethylhexyl isohexyl ester
28	14.869	239257	0.54	Undecane, 3,4-dimethyl-
29	15.137	130360	0.29	Nonane, 5-(2-methylpropyl)-
30	15.193	594150	1.34	Heptadecane
31	15.284	152185	0.34	HEXADECANE
32	15.506	632004	1.42	Phenol, 3,5-bis(1,1-dimethylethyl)-
33	15.760	214860	0.48	Nonane, 5-(2-methylpropyl)-
34	16.877	82707	0.19	Undecane, 3,8-dimethyl-
35	17.661	139622	0.31	OCTADECANE
36	17.717	422825	0.95	Eicosane
37	18.204	114323	0.26	Nonane, 5-methyl-5-propyl-
38	19.206	960408	2.16	Neophytadiene
39	19.467	14677005	32.99	Caffeine
40	19.655	327184	0.74	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
41	19.970	196535	0.44	Eicosane
42	20.395	83780	0.19	Nonane, 5-methyl-5-propyl-
43	21.938	316892	0.71	Phytol

Peak#	R.Time	Area	Area%	Name
44	22.000	188275	0.42	2-Methylhexacosane
45	22.386	104357	0.23	Decane, 1-iodo-
46	23.929	88508	0.20	2-methyloctacosane
47	28.054	133644	0.30	Carbonic acid, 2-ethylhexyl nonyl ester
48	28.754	255553	0.57	Squalene
49	33.251	1027441	2.31	Vitamin E
		44488214	100.00	

Table 5: Phytoconstituents of tea extract (Purple Tea) characterized by GC-MS

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	13.108	1707360	5.01	1,2,3-BENZENETRIOL
2	16.968	154335	0.45	Neophytadiene
3	17.224	37841	0.11	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
4	17.375	20770300	60.99	COFFEINE
5	17.883	681456	2.00	CYCLOPENTANETRIDECANOIC ACID, METHYL EST
6	19.518	42029	0.12	10,12-HEXADECADIEN-1-OL
7	19.578	291133	0.85	9-Octadecenoic acid (Z)-, methyl ester
8	19.693	75125	0.22	Phytol
9	21.220	62679	0.18	Fumaric acid, 2-dimethylaminoethyl octadecyl ester
10	21.337	164596	0.48	CHLOROMETHYL 2-CHLORODODECANOATE
11	21.498	60047	0.18	HEXADECANOIC ACID
12	21.554	47262	0.14	OXAZOLE, 2,2'-(1,4-BUTANEDIYL)BIS[4,5-DIHYDRO-
13	22.627	29738	0.09	Petroselinic acid, TBDMS derivative
14	22.677	92796	0.27	Bis(2-(Dimethylamino)ethyl) ether
15	22.820	229857	0.67	Glycidyl oleate
16	23.020	240975	0.71	Hexanoic acid, octadecyl ester
17	23.272	273553	0.80	Di-n-octyl phthalate
18	24.367	461379	1.35	(14Z)-14-TRICOSENYL FORMATE #
19	25.543	42003	0.12	Squalene
20	25.620	1627200	4.78	8-Methoxy-11-methyl-11H-indolo[3,2-c]quinoline, 5-oxide
21	26.140	247977	0.73	2-(3-METHOXY-5-METHYL-BENZYLIDENE)-7-METH
22	27.102	75379	0.22	1,3-benzenedicarboxaldehyde, 4-hydroxy-5,6-dimethoxy-
23	27.836	178786	0.52	3-Dimethylamino-6-nitro-4-phenyl-quinolin-2-ol
24	28.130	4560247	13.39	7-METHOXY-2,3,6-TRIAZAPHENOTHIAZIN-1(2H)-ON
25	28.497	136001	0.40	.gamma.-Tocopherol
26	28.755	257605	0.76	PYRAZINE, TETRAKIS(1-METHYLETHYL)-
27	29.069	410371	1.20	1-(4-Methoxybenzyl)isoquinoline
28	29.739	391353	1.15	Vitamin E
29	33.661	707104	2.08	.gamma.-Sitosterol
		34056487	100.00	

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Evolution of matK Gene among the Elite Tea Clones (*Camellia sinensis*) Revealed by Nucleotide Substitution within the Consensus Region

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ABSTRACT

The medicinally and economically important tea plant of India lacks a report on barcode study. Thus, we aimed to establish the DNA barcode of some elite tea clones of Darjeeling and Dooars along with the study of variation within the chloroplast region. A thorough investigation of 29 tea clones based on the matK (maturase K) gene has been carried out in our study. The laid objectives were fulfilled following DNA isolation, purification, amplification of the matK region, and sequencing. The sequences were further analyzed using BLAST analysis and phylogenetic tree construction along with the study of the aligned consensus region among all the clones. A BLAST search of NCBI revealed 24 clones to share 100% identity with *Camellia sinensis*. The remaining 5 clones showed 99.29–99.89% identity with *Camellia sinensis*. However, clones such as 11125 and 11126 showed a higher percentage of similarity, that is, 99.87% and 99.57% with other species of *Camellia* when compared, respectively, to 99.61% and 99.29% with *Camellia sinensis*. The relatedness to other *Camellia* species was also evident from the distinct cluster in the phylogenetic tree. This study reports a total of 14 variable sites within the matK region where the high consensus region revealed a total of nine variable sites and the low consensus region revealed a total of five variable sites. Therefore, this study is the first report of barcode analysis of Indian tea clones, wherein we successfully utilized the single locus matK gene to study variation within the chloroplast region and also conclude that the matK region is not 100% conserved with the same species of *Camellia*.

1. INTRODUCTION

The medicinally important health beverage consumed worldwide as tea, belongs to the genus *Camellia* under the Theaceae family. The world-famous tea originated in China with five reported subspecies and two varieties among which *Camellia sinensis* L.O Kuntze is mostly cultivated worldwide to make the famous tea [1]. The predominant tea varieties cultivated worldwide are the China variety with small leaves (*Camellia sinensis* L.), large leaf Assam variety (*Camellia sinensis* var. *assamica*), and intermediate leaf Cambod varieties (*Camellia assamica* var. *lasiocalyx*) [2].

Apart from its medicinal importance, tea is one of the most important cash crops of India. The world-famous Darjeeling tea serves its purpose for the Indian economy due to its unique flavor and aroma. Darjeeling tea gardens have established several elite tea clones. Environmental influences and plant age make it harder to study genetic diversity based on morphological traits unlike the molecular traits [1].

A previous study on Darjeeling tea clones reports the study of genetic diversity using a robust technique such as the RAPD, ISSR, and AFLP markers [3-5]. However, no study has been reported in the genetic diversity of Darjeeling tea clones using matK primers.

DNA barcoding, a concise method for taxonomic identification, uses a standard short sequence with ample variation to differentiate among species. Many regions from the plastid genome such as the rbcL, rpoC1, rpoB, and trnH-psbA intergenic spacer apart from the matK region have been suggested and exploited for DNA barcoding of land plants [6,7]. However, the Consortium for the Barcode of Life (CboL) has recommended rbcL and matK as standard DNA barcode for plants because of its increased variation between species and the important role it plays in the phylogenetic restoration of terrestrial plants [8,9]. The matK gene around 1500 base pairs (bp) also known as orfK is utilized in the study of molecular systematics and evolution since the matK gene contains high substitution rates within species [10]. The matK gene, coding maturase protein, is responsible for splicing of Group II intron. It is located within the intron of the trnK gene

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whose two flanking exons were lost, thus leaving the gene intact for splicing [11]. Due to its high degree of substitution and variation than other genes, the matK gene is considered to evolve quickly [12]. The matK gene has ideal size and also a mutational conserved region along with a greater rate of substitution and low transition/transversion ratio. The sequence varies at the nucleic acid level at first and second codon positions. All of these features of matK have a profound impact on relationship study at family and species level [11].

Based on previous reports, we found that the Indian tea clones have no report on barcode analysis. Thus, the main objective of the work is to study the genetic diversity and variation within the chloroplast region of the tea clones grown in Darjeeling and Dooars. We collected 33 elite tea clones from the Darjeeling region, which is famous worldwide for tea. We have chosen matK, one of the standard DNA barcodes recommended for plants and also because of the fact of the matK gene having high substitution rates within species, which makes it ideal for our study. Therefore, we aim to perform barcode analysis of collected tea clones by fulfilling steps such as genomic DNA isolation, DNA purification, and quantification, PCR amplification of the matK region, sequencing of the amplified fragments, and sequence analysis using different tools of bioinformatics. This work highlights the importance of the utilization of a single-locus matK region to study intraspecific variation and also infers the evolution of the matK gene within the same species of *Camellia*.

2. MATERIALS AND METHODS

2.1. Sample Selection and Collection

A total of 33 tea clones were collected for this study [Table 1]. The samples were collected from Darjeeling hills.

2.2. DNA Barcoding

2.2.1. DNA Isolation

DNA was isolated from a 5 g fresh leaf sample of tea clones (*Camellia sinensis*) using the CTAB extraction method with slight modification [13]. For the CTAB DNA extraction method, 5 g of fresh and tender leaves of *Camellia* was taken and pulverized using a mortar and pestle using liquid nitrogen. The pulverized material was quickly transferred and mixed into an Oakridge tube containing pre-warmed CTAB extraction buffer. It was then incubated for 1 h at 65°C with occasional mixing in-between. An equal volume of chloroform and isoamyl alcohol (24:1) was added and gently mixed. It was then centrifuged at 6000 rpm for 10 min at room temperature. An equal volume of ice-cold isopropanol was added to the supernatant and mixed by inversion. Following incubation for 2 h at −20°C, the mixture was centrifuged at 6500 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was washed thoroughly with 70% ice-cold ethyl alcohol and allowed to dry for about an hour. The pellet was dissolved in 500 µl of 1X TE buffer and to it, and an equal volume of equilibrated phenol was added and mixed properly followed by centrifugation at 13,000 rpm for 20 min. The upper aqueous layer was transferred into a fresh tube followed by the addition of an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 15 min at room temperature. The supernatant was again transferred to a new tube and treated with 1/10th volume of 3 M sodium acetate and double volume of ice-cold absolute ethyl alcohol. It was mixed gently and then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was washed using 70% ethanol, air-dried, and finally dissolved in 500 µl of 1X TE buffer.

Table 1: List of tea clones chosen for study.

SI. No	Clone	Abbreviation/Alternative name
1	Ambari Vallai 2	AV2
2	Phoobsering 312	P312
3	Happy valley 39	HV39
4	Tukdah 253	T-253
5	Nanda Devi	TS 378
6	Makaibari-6	MB-6
7	Teesta Valley 1	TTV-1
8	Tukdah 383	T-383
9	Kopati 1/1	K1/1
10	B-15/263	Badamtam -15/263
11	Balasun 7/1A/76	BS 7/1A/76
12	Bunnockburn 777	B-777
13	Sundaram	B/5/63
14	Tukdah-135	T-135
15	Tocklai seed 378	TS 378 or Nanda Devi
16	Bunnockburn 688	B-688
17	Golconda	B/6/36
18	Rungli Rungiliot 17/144	RR-17/144
19	Balasun 9/3/76	BS-9/3/76
20	Chiradew Parbat1	CP-1
21	Phoobsering 1404	P-1404
22	Phoobsering 1258	P-1258
23	Rungli Rungiliot 4/5	RR-4/5
24	Sikkim 1	SKM-1
25	Thurbo 3	Thurbo-3
26	Thurbo 9	Thurbo-9
27	Tukdah 145	T-145
28	Tukdah 246	T-246
29	Tocklai variety 19	TV-19
30	Tocklai variety 14	TV-14
31	Tukdah 78	T-78
32	Bannockburn 157	B-157
33	B/5/63	Sundaram

2.2.2. DNA purification

RNA, protein, and polysaccharides being the main contaminants in crude DNA, hamper the isolation process and it is, therefore, very important to remove such impurities. CTAB was used to eliminate polysaccharides from DNA along with subsequent use of phenol:chloroform and RNase to further eliminate proteins and RNA to a large extent from crude DNA.

For the purification process, freshly prepared RNaseA was added into the buffered solution of DNA and incubated at 37°C for 1 h in a dry bath. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed properly and centrifuged at 10,000 rpm for 15 min at room temperature. The supernatant was transferred to another tube and 1/10th volume of 3 M sodium acetate and double volume of ice-cold absolute ethyl alcohol was added followed by centrifugation at 13,000 rpm for 30 min at 4°C. Finally, the DNA pellet was washed using ice-cold 70% ethyl alcohol and air-dried and finally dissolved in 100 µl of 1X TE buffer.

Table 2: Details of matK primer.

Primer used	Tm of primer	Annealing temperature	Conc. of primer (pm/µl)	Length of primer with sequence
matK forward (F)	46	48°C	161.83	22 (CGATCTATTCATTCAATATTTC)
matK reverse (R)	53	48°C	208.38	22(TCTAGCACACGAAAGTCGAAGT)

2.2.3. DNA quantification

The isolated DNA was quantified using a UV spectrophotometer (Agilent Technologies Cary 60 UV–Vis) at 260 and 280 nm filters. The samples providing the ratio of absorbance at 260 nm to absorbance at 280 nm equivalent to 1.8 was only considered of good quality.

2.2.4. PCR amplification and sequencing of the matK region

The matK region was amplified in a 25 µl reaction volume comprising of 12.5 µl of GoTaq PCR master mix, 1.25 µl of matK-F and matK-R, 2 µl of DNA, and 8 µl of pyrogen-free water. The working concentrations taken for primers were 1.236 µM (matK-F) and 0.958 µM (matK-R), respectively. The details of the primers are given in Table 2. The PCR reactions were performed on a thermocycler (Applied Biosystems Veritti 96-well Thermal Cycle) using the following conditions: Denaturation of template DNA at 94°C for 4 min followed by 35 cycles of reactions: 94°C for 1 min, primer annealing at 48°C for 30 s, and primer extension at 72°C for 1 min with the final extension cycle at 72°C for 7 min. The success of the PCR was verified by agarose gel electrophoresis. The PCR product (5 µl) was run in an agarose gel (1%) and visualized under UV transilluminator. DNA sequencing was done using (ABI 3730 XL) from Bioserve Biotechnologies Pvt. Ltd.

2.2.5. Sequence submission

The obtained sequences were edited as per the NCBI guidelines (www.ncbi.nlm.nih.gov) and further compared by querying against existing global sequences in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using Nucleotide BLAST algorithm. Blastn and blastx were performed consecutively. The sequences were then further submitted in NCBI using the BankIt submission gateway (https://www.ncbi.nlm.nih.gov/WebSub/). The accessions number provided was retrieved from the database [Table 3].

Table 3: Accession number and details of the submitted matK sequence retrieved from NCBI.

Full name	Abbreviation	NCBI accession	Unique Id	Base pairs
Ambari Vallai 2	AV2	MH649284	1111	757 bp
Phoobsering 312	P312	MK393394	1112	871 bp
Happy valley 39	HV39	MH791417	1113	864 bp
Tukdah 253	T-253	MH920315	1114	876 bp
Nanda Devi	TS 378	MH920316	1115	816 bp
Makaibari-6	MB-6	MH920317	1116	758 bp
Teesta Valley 1	TTV-1	MH920318	1117	861 bp
Kopati 1/1	K1/1	MH920319	1119	774 bp
Balasun 7/1A/76	BS 7/1A/76	MK393393	11111	833 bp
Bunnockburn 777	B-777	MK393395	11112	757 bp
Sundaram	B/5/63	MK393396	11113	644 bp
Tukdah-135	T-135	MK393397	11114	763 bp
Bunnockburn 688	B-688	MK393398	11116	833 bp
Golconda	B/6/36	MK393399	11117	644 bp
Rungli Rungiliot 17/144	RR-17/144	MK393400	11118	826 bp
Balasun 9/3/76	BS-9/3/76	MK393401	11119	756 bp
Chiradew Parbat1	CP-1	MK393402	11120	761 bp
Phoobsering 1404	P-1404	MK393403	11121	763 bp
Phoobsering 1258	P-1258	MK393404	11122	751 bp
Rungli Rungiliot 4/5	RR-4/5	MK393405	11123	746 bp
Sikkim 1	SKM-1	MK424865	11124	867 bp
Thurbo 3	Thurbo-3	MN480321	11125	761 bp
Thurbo 9	Thurbo-9	MN480322	11126	707 bp
Tukdah 145	T-145	MK424866	11127	761 bp
Tukdah 246	T-246	MK424867	11128	756 bp
Tocklai variety 19	TV-19	MK424868	11129	750 bp
Tocklai variety 14	TV-14	MK424869	11130	735 bp
Tukdah 78	T-78	MK424870	11131	755 bp
Bannockburn 157	B-157	MK424871	11132	735 bp

2.3. Sequence Analysis

Complete 29 experimental sequences of *Camellia sinensis* clones were taken for final sequence analysis. A total of four sequences were excluded from the final dataset based on its short size (<600 base pairs) or sequencing error. The barcode sequences of the matK region of tea clones were further analyzed using MEGA X (Molecular Evolutionary Genetics Analysis) software (https://www.megasoftware.net/).

The DNA sequences were first aligned (pairwise alignment and multiple sequence alignment) using ClustalW and an evolutionary history was inferred using clustering methods such as neighbor-joining [14] and Unweighted Pair Group Mean Average method [15] with arithmetic mean (UPGMA) of MEGA X [16]. The bootstrap value was set to 1000 replicates. The phylogenetic tree drawn to scale was inferred with branch lengths in the same units as evolutionary distances. The evolutionary distances were computed using the Kimura 2- parameter method [17] with the units given as the number of base substitutions per site. The first, second, and third codon positions were included. Small sequences were not considered for analysis as well as all positions containing gaps and missing data were eliminated from the dataset. There were a total of 563 positions in the final dataset.

Further genetic pairwise distance for matK was calculated using the Kimura 2-parameter model [17] and maximum composite likelihood model [18] as given in MEGA X. Sequences were further aligned using Multalin V.5.4.1 (http://multalin.toulouse.inra.fr/ multalin/) and analysis of both high consensus and low consensus region was done. A different number of nucleotide frequencies and position of nucleotide along the consensus region were studied using Multalin (http://multalin.toulouse.inra.fr/multalin/) software V.5.4.1 [19]. Illustrative representation of the DNA barcode was done employing the sequences in matK QR code generator [20] and Biorad barcode generator (http://biorad-ads.com/DNABarcodeWeb/).

3. RESULTS

3.1. matK Amplification and Sequencing

The primer used for analysis showed successful amplification of matK [Figure 1] in all the studied clones. The size of the amplified PCR products was approximately within the range of 900 bp–1000 bp. However, the final sequencing result provided matK sequences of size ranging from 644 bp to 876 bp. The accession number for the submitted sequences and the details is provided in Table 3.

3.2. Blast Result

Blast analysis revealed 24 clones out of 29 to be 100% identical with *Camellia sinensis*. The percentage of similarity with *Camellia sinensis* recorded for other clones was 99.29% (Thurbo 9), 99.61% (Thurbo 3), 99.64% (RR-4/5), 99.88% (SKM-1), and 99.89% (P312). Despite showing 99.61% (Thurbo 3) and 99.29% (Thurbo 9) similarity with *Camellia sinensis*, both the clones showed a higher percentage of similarity with other species of *Camellia,* that is, Thurbo 3 showed 99.87% and Thurbo 9 showed 99.57% similarity with *Camellia mairei* (KJ197933.1). Thus, a percentage similarity value below 99.64% placed the clones under different species of *Camellia*.

3.3. Sequence Alignment and Phylogenetic Tree Construction

Both neighbor-joining [Online Resource 1(SM1)] and UPGMA [Figure 2] tree revealed variation among the sequences. All the combined nucleotide sequences clustered together with exceptions such as Thurbo 3 (11125), Thurbo 9 (11126) clustering together, and P312 (1112) and RR-17/144 (11118) differing from the main group. To validate our results, we also constructed a phylogenetic tree adding a sequence of different *Camellia* species (KJ197933.1) taken from the NCBI database. Thurbo 3 (11125) and Thurbo 9 (11126) now clustered with the reference sequence of *Camellia mairei* (KJ197933.1) as depicted by the neighbor- joining [Figure 3] and UPGMA tree [Online Resource 2(SM1)]).

3.4. Sequence Analysis

The genetic distances for the matK sequence ranged from 0 to 0.0090 (Nucleotide: Maximum composite likelihood method) given in Figure 4 and from 0 to 0.0089 (Nucleotide: Kimura 2-parameter method) given as Online Resource 3 (SM1). The overall mean distance was recorded as 0.0013. The results show the number of base substitutions per site and are based on an analysis of a total of 29 sequences (all codon positions included) with a total of 563 positions in the final dataset excluding the eliminated positions containing gaps and missing data.

The matK sequence showed two unique variable sites in Thurbo 3, Thurbo 9, and *Camellia mairei* that differed from the rest of the sequences. This was validated by a high consensus sequence of 563 bp prepared using Multalin software. A total of nine substitutions were observed in high consensus region where Thurbo 3 (11125) showed a total of three variable sites, Thurbo 9 (11126) showed a total of four variable sites and some single variable site was seen in P312 (1112) and RR-17/144 (11118), as shown in Figure 5 and Online Resource 4a (SM2). Study of low consensus region also revealed a total of five nucleotide substitution or variation with SKM-1 (11124) showing three variable sites, and Thurbo 9 (11126) and P1258 (11122) sowing one variable site each [Online Resource 4b (SM2)].

The sequences are represented illustratively as barcode and QR code [Figure 6 and Online Resource 5 (SM3)]. The QR code generated can be decoded as DNA sequences that make data storage and retrieval comparatively easy.

4. DISCUSSION

With the advancement of technology, sequencing analysis has uplifted the research in the molecular field, and thus, a small difference or rather variation (intraspecific or interspecific) can be studied which could not be accomplished easily using morphological means or other robust molecular techniques.

DNA barcoding is used for species identification and it utilizes many plastid and nuclear regions. A total of the seven-plastid region were explored in land plants and the combination of rbcL+matK was considered as the best combination for plant barcode [21]. Some other scientific studies reported the successful use of a combination of matK+ITS and rbcL+trnH-psbA to study 100% differences between *Cassia* species [22]. However, the efficiency of only a single matK region to differentiate *Vachellia* species from other *Acacia* species was reported earlier with concluding remarks about the possibility of utilizing matK for separating taxa at the genus level [23].

Some previous reports have suggested successful amplification and

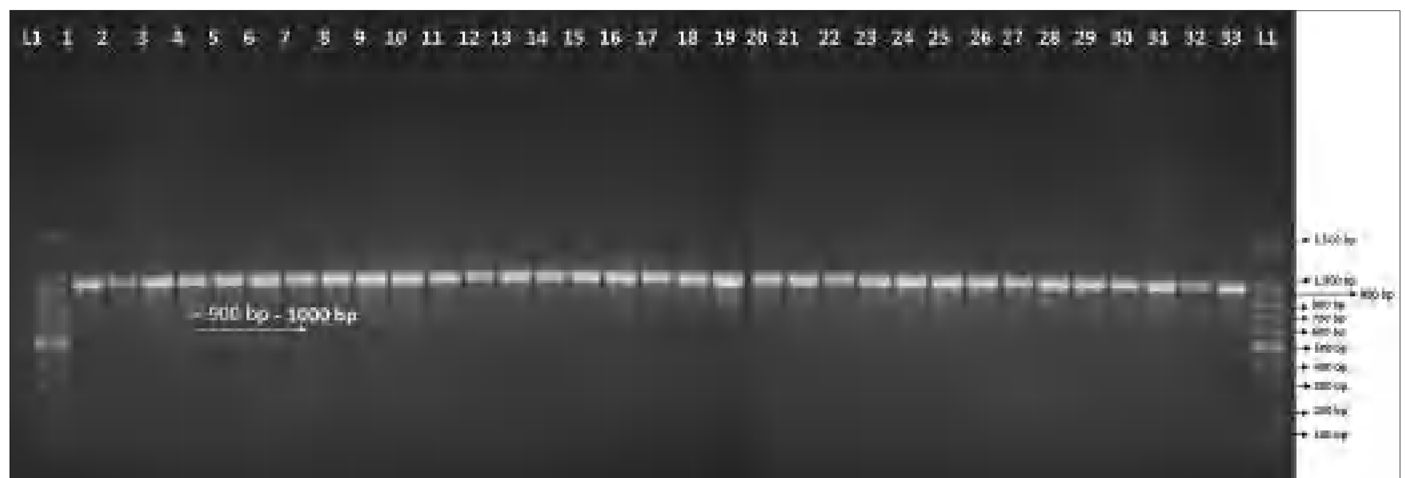

Figure 1: Amplification of the matK region. Lane L1: 100 bp ladder; lane 1–33: 33 tea clones.

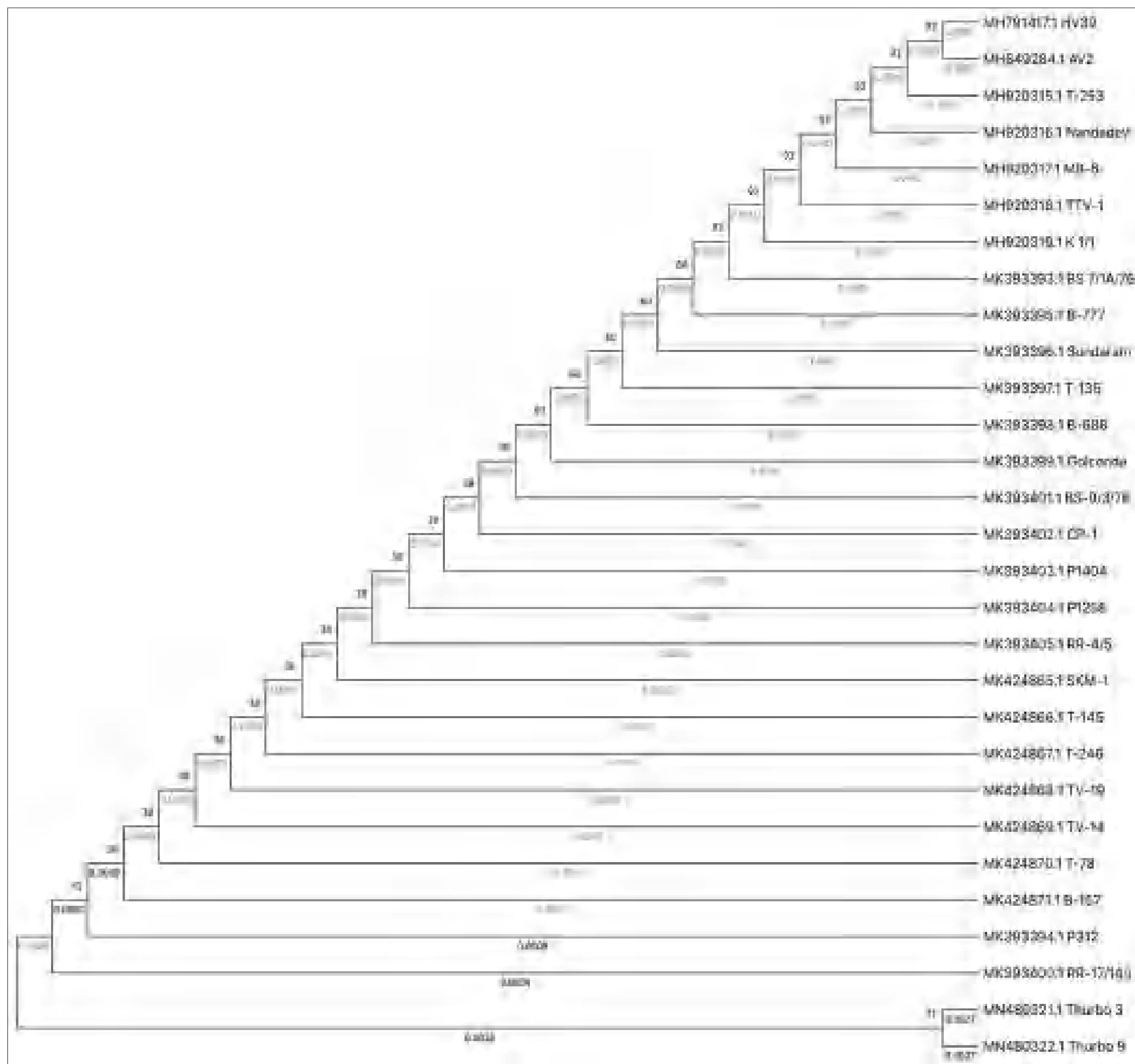

Figure 2: UPGMA tree method showing the genetic relationship of matK region between 29 tea clones.

use of the matK region to investigate phylogeny in both monocots and dicots such as *Zingiberaceae* [11], *Erythronium* [24], *Myristica fragrans* [25], local tomato [26], and oil-bearing roses [27].

The barcode technique is also used nowadays to detect any kind of contaminants. Researchers have reported the presence of adulterant with counter indications for pregnant women in bamboo tea products and also detected the origin of bamboo leaves that were used in the product [28]. Researchers have also used DNA barcodes (rbcL, matK, ITS2, and psbA-trnH) to distinguish between the commercial non-*Camellia* tea and the adulterants present in it, to assess their safety, although a limited number of original plant sequences in GenBank limited the findings [29]. There are reports of matK locus placing two genera *Myristica* and *Knena* differently at a sequence similarity of 99.43% while genus *Virola* differed with 99.25% [25]. The tomatoes were placed within the same species even with 99.64% similarity, thus limiting the assumption of percentage identity required as 99.74–100% to place organisms within the same species [30]. Whereas our study has differentiated two species at percentage identity below 99.64% with clones such as 11125 (Thurbo 3) and 11126 (Thurbo 9) showing 99.61% and 99.29% identity with *Camellia sinensis* when compared, respectively, to 99.87% and 99.57% identity with *Camellia mairei*.

Two species *T. cope* and *T. wightii* [31] did not differ at rbcl locus but showed a difference in matK (2 nucleotide difference) and trnH-psbA (1 nucleotide difference). This could broaden the interspecific variation if the two loci are considered as two-gene approach and thus they reported interspecific variation (p-distance 0.002–0.003) but

Figure 3: Neighbor-joining tree method showing the genetic relationship of matK region between 29 tea clones along with sequence of *Camellia mairei* (KJ197933.1) taken from NCBI.

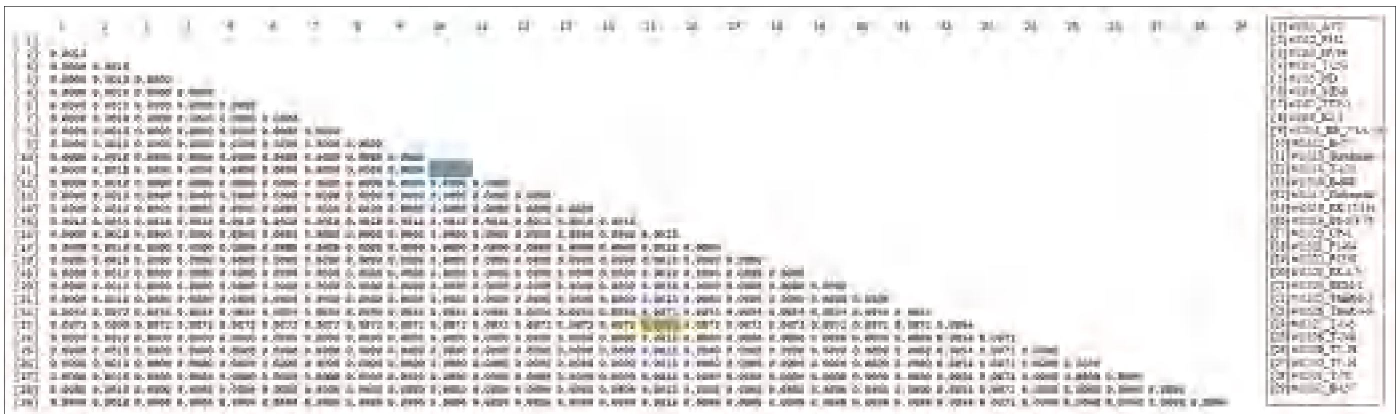

Figure 4: Genetic distances of the matK sequence calculated using nucleotide: maximum composite likelihood method.

Figure 5: Consensus region of the aligned sequences showing nucleotide substitution as highlighted by black box.

Figure 6: matK sequences represented illustratively as barcode and QR code.

no intraspecific variation (p-distance 0.00). Another work reported having three variable sites in trnH-psbA sequences among seven tomato varieties with genetic distance ranging from 0 to 0.004 [26]. On the contrary, rbcL, rpoC1, and rpoB sequences did not show any variable sites, thus suggesting it to be 100% conserved within the species. The matK locus failed to differentiate *Myristica* at species level since the blast results showed 100% similarity with other three species of *Myristica* and also reported three nucleotide differences with *Rivola sebifera* and four nucleotide difference with *Knema laurina,* thus concluding the ability of matK locus to differentiate only at the genus level within the family of Myristicaceae. However, in our study, we report a total of nine variable sites in the high consensus region and a total of five variable sites in the low consensus region of matK sequences within the same species of *Camellia sinensis*. Therefore, we report intraspecific variation and conclude with a fact of matK sequence not being 100% conserved within the same species of *Camellia*.

5. CONCLUSION

This work reports the successful use of the matK region to explore the genetic diversity and variation within the matK gene of chloroplast region among the elite clones of Darjeeling and Dooars. The employment of the matK region with its known increased rate of substitution, low transition/transversion ratio, and quick evolving rate aided to study the intraspecific variation due to probable contamination from other tea plants. The evolution of the matK region within the same species of *Camellia sinensis* was evident from our results where we report variable sites within the consensus region and conclude with the fact of the matK gene not being 100% conserved among *Camellia sinensis*. The DNA barcode of elite tea clones of Darjeeling and Dooars was thus established, wherein we conclude with the remark of matK being a good candidate for DNA barcoding of *Camellia sinensis* as well as for rapid detection of variation and molecular evidence of clones at a minimal cost, thus avoiding other robust molecular techniques.

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7. FUNDING

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8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

9. CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve the use of animals or human subjects.

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Research Article

EFFECT OF SOLVENT WITH VARYING POLARITIES ON PHYTOCHEMICAL EXTRACTION FROM MATURE TEA LEAVES AND ITS EVALUATION USING BIOCHEMICAL, ANTIMICROBIAL AND *IN-SILICO* APPROACHES

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ABSTRACT

Research in tea has been limited to processed or packaged tea. Owing to its economical as well as medicinal importance, our work is focused on phytochemical extraction from mature fresh tea leaves based on extracting solvents with varying polarities with the prime focus on bio activity study using biochemical, microbial and *in-silico* approaches. Potent solvents were screened from different qualitative, quantitative and antioxidant tests. Antimicrobial screening was done along with its validation using GC-MS and *in-silico* approaches. Acetone extracts was found to be the most potent solvent for extraction followed by methanol and ethanol. Acetone and methanol extracts showed antagonist activity against *Staphylococcus* sp. with Minimum Inhibitory Concentration of 4 mg/ml and 8 mg/ml respectively. Gas chromatography - mass spectrometry identified bioactive compounds like Phenol, 3, 5-bis (1, 1-dimethylethyl), caffeine and Vitamin E as the probable compounds as antibacterial agents. Further *in-silico* results validated phenol as the most potent antimicrobial compound with its binding affinity of -7.2 kcal/mol to *S. aureus* DNA gyrase. Elaborate qualitative and quantitative phytochemical profiling thus gave an idea about the potency of particular solvent in extracting specific group of compounds. Bioactivity of potent extract against *Staphylococcus* sp. was recorded out of other bacterial strains investigated. GC-MS analysis of extracts gave insight into the type of compounds extracted. Further *in- silico* results provided interesting insights into the ability of phenol to bind against *S. aureus* DNA gyrase.

Keywords: Polarities, Phytochemicals, Phenol, docking, gyrase

INTRODUCTION

Tea the most popular health beverage enriched with important phytochemicals belongs to the genus *Camellia* under Theaceae family. The important phytochemicals in tea includes the polyphenols (catechins and flavonoids), alkaloids (caffeine, theobromine, theophylline etc.), volatile oils, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C), inorganic elements (e.g., aluminum, fluorine and manganese etc.) with polyphenols being the most important compound of pharmacological importance[1]. It has already been proved in series of experiments that abundant polyphenols in tea imparts many health protecting activities[2]. These compounds have a wide range of pharmaceutical properties which includes anti oxidative, anti carcinogenic and anti arteriosclerotic property[3-6]. Polyphenolic compounds present in tea may reduce the risk of a variety of illnesses, including cancer, coronary heart disease, atherosclerosis, high blood cholesterol concentrations and high blood pressure. Most of the research work has been focused on made tea or processed tea putting a limitation as such to tea plant[7].

The objective of this work mainly focuses on the qualitative and quantitative phytochemical screening as well as studying the antioxidant and antimicrobial activity of fresh mature leaves of five different clones of *Camellia sinensis* based on extracting solvents of different polarities ranging from non polar to polar.

Very little work has been done on phytochemical screening of fresh leaves of tea using range of organic solvents with research being limited to standard solvents and processed tea. Extraction method should ensure complete extraction of the desired compounds of interest without any chemical modification[8]. Extraction and determination of biologically active compounds depends upon the type of solvent used where solvents will diffuse into solid plant tissue and solubilize compound with same polarity[9]. Aqueous mixtures of ethanol, methanol and acetone, water, are commonly exploited to extract plants[10]. Researchers have reported use of aqueous methanol, acetone and ethanol[6,11], absolute methanol[12], absolute ethanol[13] and boiling water for the extraction of polyphenols from green, black and mate teas[14]. Different solvents like water, aqueous ethanol in different extracting time has been employed to extract phenolics from green and white tea[15].

MATERIAL AND METHODS

Plant selection

Five tea elite clones were selected as experimental material, namely TS569 (S1), China (S2), AV2 (S3), P312 (S4) and Assam (S5), the details of which are provided in Supplementary Table 1. The samples were collected from Mirik hills of Darjeeling district and was identified by Taxonomy lab, of Botany Department, University of North Bengal (Accession no- 10339).

Solvent selection

Nine different solvents in increasing order of polarity namely hexane, benzene, chloroform, diethylether, ethyl acetate, acetone, ethanol, methanol and cold water were chosen as extracting solvents.

Sample extraction

Fresh leaves of the samples were washed thoroughly under running tap water, air-dried and then pulverized using a grinder. The sample was weighed and 3 g each was distributed equally and immersed in 30 ml each of nine different solvents ranging from non polar to polar. After 48 hours the aqueous cold extracts was centrifuged and the supernatant thus collected was dried and stored in 4°C for future use.

Qualitative screening of phytochemicals

Qualitative test for phytochemicals included test for flavonoid, tannin, steroid, terpenoid, cardiac glycoside, diterpenes, coumarin, reducing sugar, protein, and saponin with slight modification[16-18]. The method is given in Supplementary Table 2.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

As mentioned previously[19], DPPH was used to determine the antioxidant activity of the mixture of compounds extracted employing different solvents. The decrease in absorbance is marked by the free radical scavenging property of the compound, which donates hydrogen atom and scavenges the unpaired electron of the stable free radical of DPPH. To 100 µl of plant extracts (5 mg/ml) prepared from different solvents, 1900 µl of methanol was added and shaken. The mixture was incubated at room temperature for 30 minutes in dark. The absorbance was then recorded at 520 nm using spectrophotometer. Ascorbic acid was taken as a standard.
The total scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging (\%)} = \{A_{control} - A_{sample}/A_{control} \times 100\}$$

Where, $A_{control}$ denotes absorbance of only methanol and DPPH and A_{sample} denotes absorbance of sample dissolved in methanol (Plant extract/ standard) along with DPPH.

IC_{50} value was calculated using the software Kyplot 5.0. The IC_{50} value (µg/ml) is the concentration required to inhibit 50% of the initial DPPH free radical or in simple words it gives an idea about amount of extract needed for 50% inhibition. It was calculated from the graph of inhibition curve. All the reactions were monitored in triplicate and the value were expressed as the mean ± standard deviation (S.D.).

Ferric reducing power assay

Ferric reducing power assay was done as per the protocol[20] with slight modification. The antioxidant compounds act as reducers causing the reduction of Fe^{3+}/ferricyanide complex to the ferrous form which can be monitored by determining the formation of Perl's Prussian blue at 700 nm. In a test tube 250 µl of leaf extract was taken with addition of 625 µl of sodium phosphate buffer (0.2M, pH 6.6), 625 µl of $K_2\,Fe(CN)_6$ 1(% w/v) and incubated for 20 minutes at 50 °C. The tubes were then cooled and centrifuged at 3000 rpm after addition of 625 µl of TCA (10%). The upper layer of the solution or supernatant (625 µl) was mixed with equal volume of distilled water and 125 µl of $FeCl_3$ (0.1% w/v). The absorbance was finally recorded at 700 nm. Higher absorbance value indicated higher reducing power.

Quantitative estimation of total flavonoids

The total flavonoids were estimated quantitatively using $AlCl_3$ method with some modifications[21]. A total of 250 µl of sample was taken in a test tube to which 750 µl of deionized water and 75 µl of 5% $NaNO_2$ was added. Following incubation for five minutes at room temperature, 150 µl of 10% $AlCl_3$ was added. It was then incubated for six minutes at room temperature followed by addition of 500µl of 1 mM NaOH and 275 µl of deionized water. It was then incubated for 30 minutes at room temperature. The absorbance of the yellowish orange color produced by interaction of flavonoid with $AlCl_3$ was recorded at 510 nm using spectrophotometer. Quercetin was taken as a standard and the total flavonoid content was calculated by taking reference from a calibration curve (y = 0.2071x - 0.2048) of quercetin taken at different concentrations (1-5 mg/ml) [Supplementary Figure 1(a)]. The total flavonoids were thus expressed as mg of quercetin equivalent per g of extract i.e., mg QE/ g. Data was expressed as mean of triplicates ± standard deviation.

Quantitative estimation of total phenol

The total phenolic content of the sample was determined using the Folin – Ciocalteu method[22] with slight modification. 100 µl samples were taken in a test tube and to it 400 µl of 10 % Folin reagent was added (1 ml Folin + 9 ml distilled water). The mixture was incubated in dark for 5 minutes at room temperature followed by addition of 1 ml of 5% Na_2CO_3. After incubating it for 2 h in dark at room temperature, absorbance was recorded at 730 nm using spectrophotometer. Gallic acid was taken as a standard and the total phenol content was calculated by taking reference from a calibration curve (y = 0.0075x - 0.0252) of gallic acid [Supplementary Figure 1(b)] taken at different concentrations (50-300 µg/ml). The total phenols in the extract were thus expressed as mg of gallic acid equivalent per gram of extract (mg GAE/g). Data was expressed as mean of triplicates ± standard deviation.

Antimicrobial activity

Antimicrobial activity of the acetone and methanol extracts of different tea clones was carried by agar well diffusion method[23-25]. Four different bacterial strains were employed out of which two were gram positive bacteria (*Staphylococcus* sp. and *Bacillus* sp.) and the other two were gram negative bacteria (*Escherichia coli* and *Klebsiella* sp.).

Agar well diffusion method

Sterile petri plates of 90 mm were taken to which 25 ml of MHA (Muller Hinton agar) media was poured under aseptic conditions. After the solidification of the media 45 µl of bacterial strains was pipetted into each plate and swabbed or spread uniformly using a sterile cotton swab. A well was punctured into the solidified agar using a sterile micro tip. To each agar wells 100 µl of plant extracts (10 mg/ml) was pipetted along with a sterility control (DMSO) and a positive control (Streptomycin sulphate: a broad spectrum antibiotic used as a standard). The concentration of the standard taken was 10 folds lower than the acetone and methanol extracts. The experimental set up was same for all the samples which were performed in duplicates. The plates were incubated at 37°C for 24 hours or 48 hours if needed. The antimicrobial activity was recorded with reference to appearance of clear halo or inhibition zone on the plates.

MIC (Minimal Inhibitory Concentration) determination

The MIC value was further determined only for *Staphylococcus* sp. using agar well diffusion technique as mentioned above. Different concentrations were taken i.e., 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 4 mg/ml, 8 mg/ml and 10 mg/ml respectively. 100 µl of each dilution rendering different concentrations was introduced into the wells of MHA (Muller Hinton Agar) plates in duplicates which were pre-inoculated with *Staphylococcus* sp. where DMSO was used as a control. The least concentration of the acetone or methanol extract of sample, showing a clear zone of inhibition was considered as its MIC[25].

GC-MS analysis

The acetone extracts of the sample S1 (TS-569) and S3 (AV2) was further employed for GC-MS analysis. The GC-MS analysis was performed using a GCMS-QP2010 Plus (Shimadzu Corporation, Kyoto Japan) the details of which are discussed in previous reports[26].

***In-silico* analysis**

DNA gyrase is one of the Type II topoisomerase relieving strain while ds DNA is unwounded by RNA polymerase or by helicase specifically at replication fork. It either introduces negative supercoiling or relaxes the positive supercoiling and thus help in the initiation and progression of DNA replication[27]. Hence gyrase may be regarded as one of the major house-keeping proteins among organisms. This fact has been used in the docking study. Crystal structure of *S. aureus* DNA gyrase (pdb id: 5CDN) was downloaded from PDB database (http://www.rcsb.org/structure/5CDN). The resolution of the structure was 2.79 Å and was obtained through X-ray diffraction method. This crude structure was polished by removal of water and addition of polar hydrogen bonds. The dimensions of grid box were taken in such a way that the whole protein could be considered for searching the best binding site and finally the protein structure was ready for docking. The structure of Phenol was downloaded from NCBI Pubchem database. The physiochemical properties of phenol were taken into consideration. Molecular docking was done in Auto dock Vina software and was visualized via Pymol software.

Statistics

The readings were taken in triplicates and data was represented as mean ± sd. Statistical analysis relating to t- test for paired comparison of mean was performed employing KyPlot software (version 5.0). Results were considered significant at level of significance $p \leq 0.001$.

RESULTS

Qualitative test

Acetone, methanol, and ethanol persistently proved to be the most potent solvent for various phytochemical extractions as it gave positive result for tests like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin, cardiac glycoside, saponin and reducing sugar (Figure 1). The result varied to some extent in different clones and solvents since traces of phytochemicals named above were also found in extracts prepared from, diethylether, ethylacetate, and cold water as shown in the heatmap (Figure 1). However traces of phytochemicals like cardiac glycoside, flavonoid and tannin were found in extracts of less polar solvents like benzene, chloroform and hexane of seed clones S1 and S2.

The antioxidant ability of the sample cannot be concluded only by one method[28], so we used methods to estimate total phenols and flavonoids quantitatively, and also performed DPPH free radical scavenging assay and FRP assay.

DPPH assay

The highest percentage of radical scavenging (Supplementary Figure 2) at single concentration 200 µg/ml was recorded in acetone extracts (200 µg/ml) i.e. 71.90% followed by methanol (75.31%) and ethanol (73.90%) against ascorbic acid used as standard (94.82%). Overall, the lowest scavenging activity was seen in benzene extracts. Acetone and methanol being the best solvents to extract compounds with greater antioxidant potential was further compared with ascorbic acid (standard) at various concentrations ranging from 1 mg/ml to 5 mg/ml. Both the solvent extracts i.e. acetone Figure 2(a) and methanol Figure 2(b) showed antioxidant potential or rather free radical scavenging activity at par with the standard (ascorbic acid). However, acetone gave more promising result comparatively in almost all clones. Regarding samples, acetone extracts of S1 and methanol extracts of S2 showed better antioxidant potential compared to other clones with lowest IC_{50} value being 0.111 ± 0.001 mg/ml and 0.478 ± 0.028 mg/ml respectively. The IC_{50} value of standard (ascorbic acid) was recorded as 0.057 ± 0.000 mg/ml (Table 1).

Ferric reducing power assay

Similarly, acetone extracts Figure 2(c) showed higher ferric reducing power in almost all the clones than in methanol Figure 2(d) with S1 and S2 mostly giving better results. The ferric reducing power was almost at par with the ferric reducing power of ascorbic acid used as standard.

Total Phenol

Acetone extracts gave the best result overall along with the highest value of phenol recorded in acetone extracts of China seed variety (S2) as 37.77 ± 1.28 mg GAE/g. Next to acetone, methanol and ethanol gave persistently better results (Table 2). The lowest value recorded was that of chloroform extracts of S2 i.e. 0.25 mg GAE/g of total phenols.

Total flavonoid

The highest value of 722.94 ± 127.01 mg QE/g was recorded for methanolic extracts of S1 (Table 3). The lowest value was recorded for hexane extracts of S3 as 399.42 ± 2.73 mg QE/g.

Antimicrobial screening

From the antimicrobial activity studied, acetone and methanol extracts were found to be more effective and bactericidal against *Staphylococcus* sp. Figure 3 (1) and on the other hand minimum or negligible activity was observed against other bacterial strains. When compared with the standard, pipetting more extracts into the well proved beneficial as the volumetric increase of plant extracts gave results almost at par with the 100 µl of the standard Figure 3(2). The MIC of the acetone extracts of tea clones was found to 4 mg/ml Figure 4 (1) whereas for methanol extracts the MIC was recorded as 8 mg/ml Figure 4 (2).

Gas chromatography- mass spectrometry analysis

Further analysis of the extracts using Gas chromatography- mass spectrometry identified bioactive compounds like Phenol, 3,5-bis(1,1-dimethylethyl), caffeine and Vitamin E as the probable compounds as antibacterial agents in both the extracts of S1

(Supplementary Figure 3 ; Supplementary Table 3) and S3 (Supplementary Figure 4; Supplementary Table 4).

In-silico results

The binding affinity of phenol to 5CDN was found to be -7.2 kcal/mol which showed the significant effect of phenol with the gyrase protein (Figure 5). The other two compounds did not show any effective binding.

DISCUSSION

The nature of extracting solvent plays an important role in extraction of potential compounds of antioxidant activity since the compounds differ in chemical characteristics, polarities and solubilities[29]. Presence of alkaloids, flavonoids, saponins, terpenoids and phenols were reported in plant extracts of *Camellia sinensis* (purple tea) and the solvents with higher polarity i.e. water, ethanol and acetone were found to extract major phytochemicals groups than non-polar ethyl acetate and chloroform[30]. Methanol showed better extraction properties than acetone and ethyl acetate for extracting few phytochemicals like flavonoid, tannin, triterpenes, lipid and reducing sugar in black packaged tea where other solvents showed minimum activity[28]. The polar solvents and in some cases even the least polar solvents showed best result in extracting phytochemicals from fresh leaves of seed clones like S1 and S2 and we could therefore infer that in addition to extraction of samples using solvent with different polarities, extraction time and procedure, the state of sample also matters in phytochemical extraction since the phytochemical constituent slowly degenerates from the time of plucking up to manufacturing. Qualitative screening of phytochemicals is important to the pharmaceutical industry since the presence of a phytochemical of interest may lead to its further isolation, purification and characterization[31].

Table 1: **IC50 value of acetone and methanol extracts prepared in different concentrations (mg/ml) during DPPH Assay**

Accession	IC_{50} (mean ± sd) of acetone extracts (mg/ml)	IC_{50} (mean ± sd) of methanol extracts (mg/ml)
S1 (TS569)	0.111 ± 0.001	0.635 ± 0.028
S2 (China)	0.159 ± 0.002	0.478 ± 0.028
S3 (AV2)	0.600 ± 0.002	1.030 ± 0.061
S4 (PS312)	0.294 ± 0.008	1.595 ± 0.297
S5 (Assam)	0.515 ± 0.002	1.016 ± 0.039
ST (Ascorbic acid)	0.057 ± 0.000	0.056 ± 0.000

Data expressed as means of triplicates ± sd

Table 2: Determination of total phenol content (TPC) expressed as mg Gallic acid equivalent (GAE) /g in fresh leaves tea clones extracted by different solvents

Solvents	S1 Mean ± sd (mgGAE/g)	S2 Mean ± sd (mgGAE/g)	S3 Mean ± sd (mgGAE/g)	S4 Mean ± sd (mgGAE/g)	S5 Mean ± sd (mgGAE/g)
Hexane	1.61 ± 0.08	0.60 ± 0.75	3.66 ± 3.51	1.00 ± 0.04	1.08 ± 0.38
Benzene	2.14 ± 0.98	0.38 ± 0.00	2.86 ± 0.79	1.21 ± 0.04	1.50 ± 0.08
Chloroform	1.77 ± 0.08	0.25 ± 0.19	1.50 ± 0.00	1.45 ± 0.08	1.85 ± 0.26
Diethylether	3.42 ± 0.15	7.18 ± 0.72	2.49 ± 0.26	2.14 ± 0.00	3.34 ± 0.11
Ethylacetate	6.41 ± 0.15	5.40 ± 0.68	3.77 ± 0.11	3.69 ± 0.08	5.42 ± 0.26
Acetone	32.44 ± 0.98	37.77 ± 1.28	32.06 ± 0.75	14.57 ± 1.66	18.65 ± 2.07
Ethanol	18.25 ± 0.15	16.92 ± 0.98	17.88 ± 0.60	14.25 ± 4.75	13.72 ± 0.08
Methanol	32.89 ± 7.96	31.50 ± 9.99	23.82 ± 6.45	10.73 ± 0.15	11.50 ± 1.24
Water	4.04 ± 0.19	1.53 ± 0.11	2.36 ± 0.53	2.52 ± 0.38	2.25 ± 0.30

$y = 0.0075x-0.0252$, $R^2 = 0.9864$. Data expressed as means of triplicates ± sd

Table 3: Determination of total flavonoid content (TFC) expressed as mg Quercetin equivalent (QE)/g in fresh leaves of tea clones extracted by different solvents

Solvents	S1 Mean ± sd (mgQE/g)	S2 Mean ± sd (mgQE/g)	S3 Mean ± sd (mgQE/g)	S4 Mean ± sd (mgQE/g)	S5 Mean ± sd (mgQE/g)
Hexane	412.94 ± 8.19	446.74 ± 23.22	399.42 ± 2.73	418.73 ± 13.66	537.52 ± 28.68
Benzene	406.18 ± 1.37	441.91 ± 24.58	488.27 ± 73.75	401.35 ± 0.00	463.16 ± 5.46
Chloroform	428.39 ± 2.73	482.47 ± 19.12	412.94 ± 2.73	467.02 ± 10.93	477.64 ± 15.02
Diethyl ether	467.99 ± 12.29	473.78 ± 36.87	433.22 ± 1.37	448.67 ± 4.10	561.66 ± 16.39
Ethyl acetate	470.88 ± 5.46	507.58 ± 10.93	433.22 ± 4.10	424.53 ± 8.19	469.92 ± 23.22
Acetone	513.76 ± 136.03	675.62 ± 51.90	617.67 ± 32.78	579.04 ± 8.19	669.82 ± 87.41
Ethanol	528.83 ± 19.12	557.80 ± 8.19	570.35 ± 6.83	506.62 ± 9.56	611.88 ± 16.39
Methanol	722.94 ± 127.01	581.94 ± 17.75	545.24 ± 45.07	519.17 ± 35.51	574.22 ± 1.37
Water	424.53 ± 10.93	406.18 ± 6.83	432.25 ± 10.933	423.56 ± 34.14	431.29 ± 4.10

$y = 0.2071x - 0.2048$, $R^2 = 0.9625$. Data expressed as means of triplicates ± sd

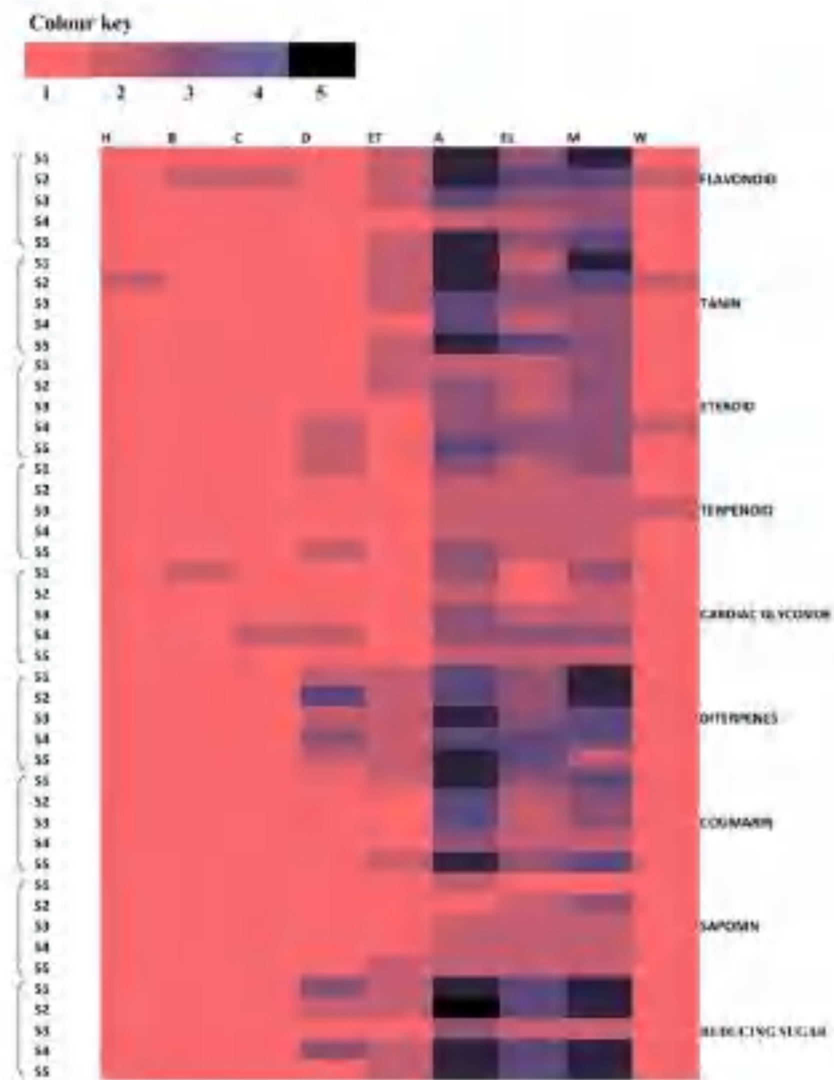

Figure 1: Heat map representing the qualitative phytochemical profiling of different extracts of selected tea clones

Colour key provided from pink to black represents the intensity in increasing order. H-Hexane, B-Benzene, C-Chloroform, D-Diethyl ether, ETEthyl acetate, A-Acetone. EL-Ethanol, M-Methanol, W-Water

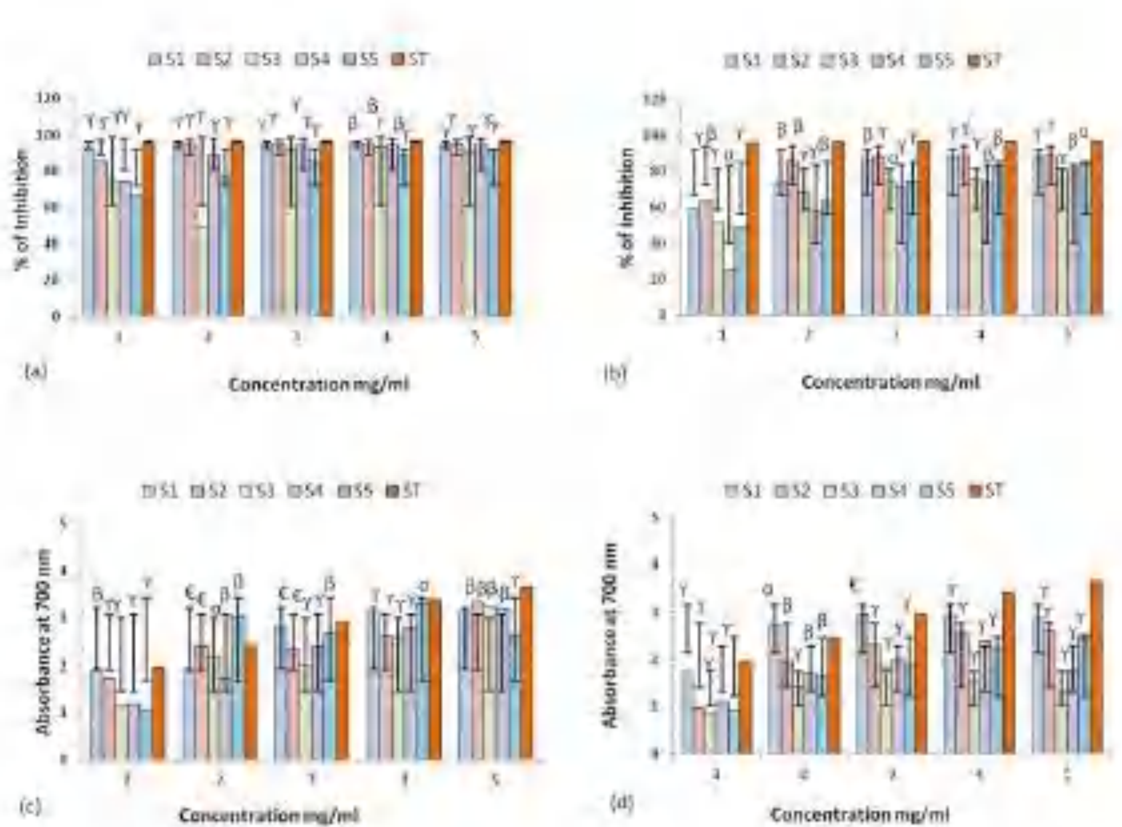

Figure 2(a): DPPH scavenging activity of acetone extracts; (b): DPPH scavenging activity of methanol extracts; (c): Ferric reducing power of acetone extracts (d): Ferric reducing power of methanol extracts;
$^{\alpha}$ $p < 0.05$; $^{\beta}p < 0.01$; $^{\gamma}p < 0.001$; $^{\epsilon}$non significant

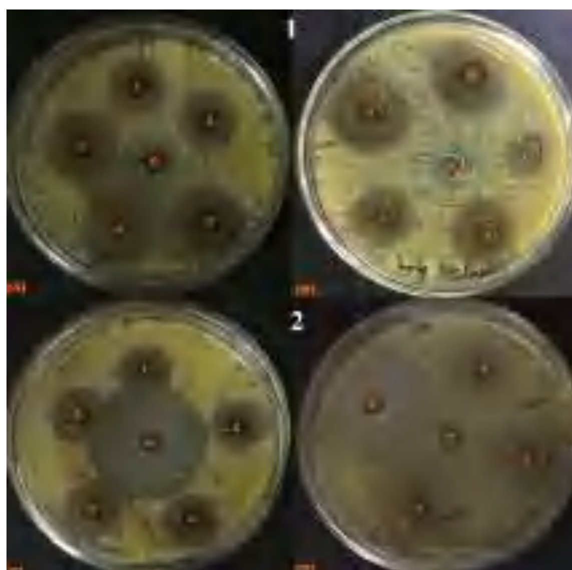

Figure 3(1): Antimicrobial activity of tea extracts against *Staphylococcus* sp.
(A) Acetone (B) Methanol, 1-5 different tea clones; C sterility control (DMSO); (2) Antimicrobial activity of tea extracts against *Staphylococcus* sp. (C) Acetone extract (100 µl of 10 mg/ml) of five different tea clones with standard; 1-5 different five clones; S positive control (Streptomycin sulphate), (D) Acetone extract of S1 with standard; 1-3 different volumetric dose; 1– 100 µl , 2– 200 µl, 3– 300 µl, S positive control (Streptomycin sulphate); C sterility control DMSO

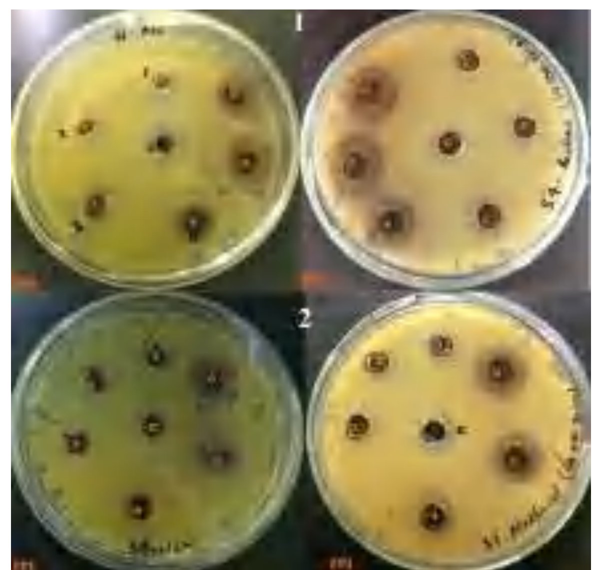

Figure 4(1): MIC value determination from antimicrobial activity of tea extracts against *Staphylococcus* sp. using agar well diffusion method (A) Acetone extracts of S2 (B) Acetone extracts of S4; 1-5 different concentrations 1-0.25 mg/ml, 2– 05 mg/ml, 3– 1 mg/ml, 4– 4 mg/ml, 5– 8 mg/m, 6– 10 mg/ml; C sterility control (DMSO); (2) MIC value determination from antimicrobial activity of tea extracts against *Staphylococcus* sp. using agar well diffusion method (C) Methanol extracts of S4(D) Methanol extracts of S7; 1-5 different concentrations 1-0.25 mg/ml, 2– 05 mg/ml, 3– 1 mg/ml, 4– 4 mg/ml, 5– 8 mg/m, 6– 10 mg/ml; C sterility control DMSO

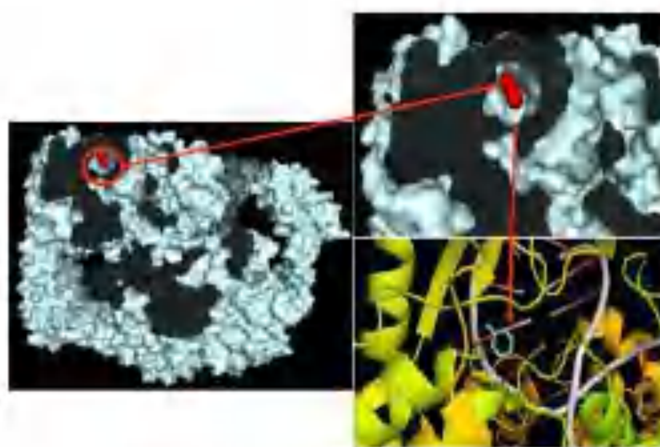

Figure 5: *In-silico* docking of *S. aureus* DNA gyrase (pdb id: 5CDN) showing significant binding affinity -7.2 kcal/mol with phenol

Methanol[28] ethanol and acetone extract[14] has already been reported to have strong antioxidant property in black tea. Previous report[14] highlights the varying antioxidant activity with changes in percent concentration of the solvent thus reporting 50% ethanol and 50% acetone with maximum antioxidant potential in mate tea and black tea respectively and also showed the capability of hot water showing moderate antioxidant potential in black tea and higher antioxidant potential in mate tea when compared to other 100% solvent. Thus it could be inferred that solvent potential may be enhanced or reduced with altering the percent concentration of the solvent.

The IC_{50} value calculated represents the exact concentration for 0.5 absorbance at 700 nm and the reducing power mostly increases with increasing concentration of antioxidant compounds[28,32,33]. Previous report on fresh tea leaf sample[34] has stated methanol extract showing highest reducing power based on state of sample where greater ferric reducing power was showed by shoot extract followed by young leaves and mature leaves.

The minimum level of phenols was recorded in less polar solvent extracts like hexane, benzene, and chloroform. However, the consistency of better result did not limit to the range of polarity in increasing or decreasing order. It varied among the different sample as well as solvents. It has already been reported in previous works that green tea was a richer source of phenolics than white tea and found 40% aqueous ethanol to be useful for extracting catechin[15]. Total phenol content estimation following Folin-Ciocalteu reagent method showed 50% acetone, 50% N, N-dimethylformamide (DMF), 50% ethanol and 50% methanol to be suitable for extracting total phenols[14].

Previously aqueous tea extracts were found to be effective against range of bacterial strains including *Staphylococcus aureus* with bactericidal activity against *Staphylococcus aureus* and *Yersinia enterocolitica*[35]. However in our study significant antimicrobial activity was found for acetone and methanol extract against *Staphylococcus* sp. with minimum or negligible activity against other strains.

Vitamin E being a powerful antioxidant showing scavenging as well as anti-inflammatory properties was also found to be effective against gram positive bacteria than gram negative ones owing to the fact of lipopolysaccharides present in their outer membrane[36]. On the other hand, the antimicrobial activity of *Arabica coffee* extracts against *Staphylococcus epidermidis* and *Enterococcus faecalis* was found to be independent from caffeine content[37]. However, caffeine was found to potentiate or enhance the antibacterial activity of amoxicillin against *Staphylococcus aureus*[38]. Significant inhibition of hexane extract of *Ulva reticulata* against *Staphylococcus aureus* and *Escherichia coli* reported presence of phenol, 3, 5-bis (1, 1-dimethylethyl) in GC-MS analysis as the probable compound giving antibacterial activity[39].

Phenol is a well-known antimicrobial agent with both bacteriostatic and bactericidal effect. Phenol may exert its effect on external membrane of bacterial cell wall, cytoplasmic membrane, organelles, cytosol as well as on spores[40]. The penetrating power of phenol into organic matter is quite high and active phenol may enter through bacterial cytoplasmic membrane via passive diffusion and active transport. Moreover, phenol acts as strong oxidizing agent and converts akin to oxide and gas, which ultimately destabilize the spores[40]. Thus, the binding of phenol to gyrase a protein may also exert as strong antimicrobial effect.

CONCLUSION

Acetone, methanol, and ethanol showed persistent and better results to extract various phytochemicals like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin, cardiac glycoside, saponin, and reducing sugar. Elaborate qualitative phytochemical profiling thus gave an idea about the potency of particular solvent in extracting specific compound of interest. Acetone extracts gave the best result overall during radical scavenging assay, ferric reducing power assay, total phenol and flavonoid estimation followed by methanol. Out of the four bacterial strain studied the acetone extracts along with methanol extracts was found effective against the *Staphylococcus* sp. with the MIC value 4 mg/ml for acetone extracts and 8 mg/ml for methanol extracts respectively. GC-MS and *in- silico* analysis suggested phenol 3, 5-bis (1, 1-dimethylethyl) as the potent antimicrobial compound. Thus, it can be concluded that abundancy of phytochemicals may vary from one clone to the other or its state (either fresh, dry or manufactured), beside varying time and procedure and the type of solvent utilized for extraction. We could also infer from our results that there isn't any pattern of potentiality of solvents in increasing or decreasing order. The collective information of this work could promote future research to utilize resources in an efficient manner.

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Effect of solvents on qualitative and quantitative phytochemical constituent profiles of fresh leaves of TV26

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Abstract
The aim of this study was to qualitatively and quantitatively screen and identify major phytochemical groups from leaves extract of TV26 extracted by nine different solvents having different polarities. Qualitative screening suggested acetone, methanol, ethanol and ethyl acetate to be the most potent solvent for various phytochemical extractions like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin, cardiac glycoside, saponin, protein and reducing sugar. The highest percent of radical scavenging was recorded in cold water extracts (5mg/ml) i.e. 91.10% and was at par with 93.40% percent scavenging activity of ascorbic acid taken as standard (5mg/ml). Methanol, acetone, ethanol and ethyl acetate gave the best results with the total phenol content value (GAE) recorded as 100.60mg/g, 87.07mg/g, 58.73 mg/g and 51.47mg/g respectively with methanol giving the best result. Acetone extracts (5mg/ml) showed higher ferric reducing power with IC_{50} value 426.45±1.12 µg/ml compared to the standard (ascorbic acid) 270.35±0.66 µg/ml. Our findings suggest that the polar solvents were more beneficial and potent against the other non polar counterparts during phytochemical extraction but the polarity of solvents need not be in increasing order since we can assume from our results that acetone being less polar than ethanol, methanol and water showed better results. In addition to different polarities, state of the sample and extraction technique is also crucial for better extraction.

Keywords: TV26, Phytochemicals, Solvent, Qualitative, Quantitative.

Tea, the most popular health beverage next to water in the world, has aroused great interest among the world of scientific research due to its beneficial health effects. It belongs to the genus *Camellia* under Theaceae family with, *Camellia sinensis* (L.)O. Kuntze being the mostly used species for making the health beverage (Kaundun *et al.* 2000). The important phytochemicals in tea includes the polyphenols (catechins and flavonoids), alkaloids (caffeine, theobromine, theophylline etc.), volatile oils, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C), inorganic elements (e.g., aluminium,fluorine and manganese etc.) with polyphenols being the most important compound of pharmacological importance (Sharangi 2009). It has already been proved in series of experiments that abundant polyphenols in tea imparts many health protecting activities (Manzocco *et al.* 1998). These compounds have a wide range of pharmaceutical properties which includes antioxidative, anticarcinogenic and antiarteriosclerotic (Atoui *et al.* 2005; Dufresne and Farnworth 2001; Filip and Ferraro 2003; Wang and Helliwell 2001). Polyphenolic compounds present in tea may reduce the risk of a variety of illnesses, including cancer, coronary heart disease, atherosclerosis, high blood cholesterol concentrations and high blood pressure. Most of the research work has been focused on manufactured tea or processed tea putting a limitation as such to tea plant (Mukhtar and Ahmad 2000).

Varied range of solvents has been used for extracting polyphenols from plants (Chavan *et al.* 2001) and the role of extracting solvents and the method of extraction on total extraction yield has already been highlighted in several articles (Goli *et al.* 2005). Extraction method should ensure complete extraction of the desired compounds of interest without any chemical modification (Zuo *et*

al. 2002). Extraction and determination of biologically active compounds depends upon the type of solvent used where solvents will diffuse into solid plant tissue and solubilize compound with same polarity (Tiwari *et al*. 2011).

Aqueous mixtures of ethanol, methanol and acetone, water, are commonly exploited to extract plants constituents (Sun and Ho 2005). Researchers have reported use of aqueous methanol, acetone and ethanol (Martinez *et al*. 1997; Wang and Helliwell 2001), absolute methanol (Yao *et al*. 2004), absolute ethanol (Opie *et al*. 1990) and boiling water for the extraction of polyphenols from green, black and mate teas (Turkmen *et al*. 2006). Different solvents like water, aqueous ethanol in different extracting time has been employed to extract phenolics from green and white tea (Rusak *et al*. 2008).

Very little research has been done on phytochemical screening of tea using range of organic solvents. Research has been mostly limited to standard solvents and processed tea thus limiting the study of different phytochemicals in fresh leaves of tea extracted by range of organic solvents. Therefore our study mostly focused on preliminary area of research covering the qualitative and quantitative phytochemical screening as well as studying the antioxidant activity of fresh leaves of TV26 based on extracting solvents of different polarities. The range of organic solvent system with different polarities included absolute hexane, benzene, chloroform, diethylether, ethylacetate, acetone, ethanol, methanol and water.

Materials and methods

Chemicals

10% $FeCl_3$, 1% $FeCl_3$, chloroform, concentrated H_2SO_4, glacial acetic acid, 5% copper acetate, 10%NaOH, Benedict reagent, concentrated HNO_3, distilled water, 2,2-diphenyl-1-picrylhydrazyl, Hexane, Benzene, Diethylether, Ethylacetate, Acetone, Ethanol, Methanol and Water, sodium phosphate buffer (0.2M, pH 6.6), K2 $[Fe(CN)_6]$ 1(% w/v), TCA (10%), $FeCl_3$ (0.1% w/v), Folin reagent, 5% Na_2CO_3, gallic acid, ascorbic acid.

Variety selection

TV26 (Tocklai variety) was chosen as the experimental variety and leaves were collected from tea garden of University of North Bengal. TV26 is a variety of Cambod origin with average quality, high yield and is moderately tolerant to drought (http://www.tocklai.net/activities/tea-cultivation/tra-garden-series-clones/).

Sample extraction

Fresh leaves of TV26 collected were washed thoroughly under running tap water, air dried and then pulverized using a grinder. The sample was weighed and 3g each was distributed equally and immersed in 30 ml each of nine different solvents ranging from polar to non polar. Nine different solvents in increasing order of polarity namely hexane, benzene, chloroform, diethylether, ethylacetate, acetone, ethanol, methanol and water were chosen as extracting solvents. After 48 hours the aqueous cold extracts was centrifuged and the supernatant thus collected was dried and dissolved in methanol. The aliquots of different aqueous extract were thus stored at room temperature.

Qualitative screening of phytochemicals

Qualitative test for phytochemicals included test for flavonoid, tannin, steroid, terpenoid, cardiac glycoside, diterpenes, coumarin, reducing sugar, protein, and saponin with slight modification (Brain and Turner 1975; Kumar *et al*. 2009; Ngbede *et al*. 2008).

Flavonoid

To 250µl of sample few drops of 10% $FeCl_3$ was added to which a blue or green coloration confirmed the presence of flavonoids .

Tanin

Appearance of blue or green colour formation on addition of few drops of 1% $FeCl_3$ to 250 µl of extract confirmed the presence of tannins.

Steroid

About 250 µl of extract was evaporated and dissolved in 2ml of chloroform and about 2ml of concentrated H_2SO_4 was added from the sidewall of the test tube. Appearance of reddish brown colour ring confirmed the presence of steroids.

Terpenoid

The evaporated extract (250 µl) was dissolved in chloroform and concentrated H_2SO_4 was added from the sidewall of test tubes and then shaken.

Formation of red to reddish brown coloration at the base confirmed the presence of terpenoids.

Cardiac glycoside

250 µl of extract was evaporated and to it 1ml of glacial acetic acid, one drop of 10% $FeCl_3$ and 1 ml of concentrated H_2SO_4 was added. A brown ring at the interface indicated the presence of cardiac glycosides.

Diterpenes

Copper acetate was performed to confirm the presence of diterpenes wherein addition of few drops of 5 % copper acetate to 250 µl of extract dissolved in equal volume of distilled water formed emerald green color.

Coumarin

Yellow coloration on addition of about 500 µl of 10% NaOH to 250 µl of sample confirmed the presence of Coumarins.

Reducing sugar

Benedict test was performed to estimate the presence of reducing sugar in the extracts wherein 1ml of Benedict reagent added to 250 µl of sample gave green coloration (color varies from green to red depending upon the percentage of reducing sugar present).

Protein

Xanthoproteic test was performed where 1ml concentrated HNO_3 was added to about 250 µl sample thus giving a yellow precipitate.

Saponin

Froth test was conducted to confirm the presence of saponin with appearance as well as persistence of froth while shaking the sample diluted with distilled water.

Quantitative screening of Phytochemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

As mentioned by (Blois 1958), DPPH was used to determine the antioxidant activity of the mixture of compounds extracted employing different solvents. The decrease in absorbance is marked by the free radical scavenging property of the compound, which donates hydrogen atom and scavenges the unpaired electron of the stable free radical of DPPH. To 100 µl of plant extracts (5mg/ml) prepared from different solvents, 1900 µl of methanol was added and shaken. The mixture was incubated at room temperature for 30 minutes in dark. The absorbance was then recorded at 520 nm using spectrophotometer. Ascorbic acid was taken as a standard.

The total scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging (\%)} = \{A_{control} - A_{sample}/A_{control} \times 100\}$$

Where, $A_{control}$ denotes absorbance of only methanol and DPPH and A_{sample} denotes absorbance of sample dissolved in methanol (Plant extract/ standard) along with DPPH.

Folin-Ciocalteu reagent method for determination of phenol

The total phenolic content of the sample was determined using the Folin – Ciocalteu method (Folin and Ciocalteu 1927) with slight modification. 100 µl samples were taken in a test tube and to it 400 µl of 10 % Folin reagent was added (1ml Folin + 9 ml distilled water). The mixture was incubated in dark for 5 minutes at room temperature followed by addition of 1 ml of 5% Na_2CO_3. After incubating it for 2 hrs in dark at room temperature, absorbance was recorded at 730 nm using spectrophotometer. Gallic acid was taken as a standard and the total phenol content was expressed in mg of GAE per g of extract or GAE mg/g.

Ferric reducing power assay

Ferric reducing power assay was done as per the protocol (Aiyegoro and Okoh 2009) with slight modification. In a test tube 250 µl of leaf extract was taken with addition of 625 µl of sodium phosphate buffer (0.2M, pH 6.6), 625 µl of K2 $[Fe(CN)_6]$ 1(% w/v) and incubated for 20 minutes at 50 °C. The tubes were then cooled and centrifuged at 3000 rpm after addition of 625 µl of TCA (10%). The upper layer of the solution or supernatant (625 µl) was mixed with equal volume of distilled water and 125 µl of $FeCl_3$ (0.1% w/v). The absorbance was finally recorded at 700nm. Higher absorbance value indicated higher reducing power.

Results and Discussion

Acetone, methanol, ethanol and ethyl acetate persistently proved to be the most potent solvent for various phytochemical extractions as it gave

positive result for various test like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin,cardiac glycoside, saponin, protein and reducing sugar (Table1, Fig. 1). However, extraction of phytochemicals varied in some solvents. Methanol, cold water, ethanol, and ethylacetate proved to be the best solvents for extracting cardiac glycosides. Traces of steroid, diterpenes, reducing sugar, saponin was found in diethylether extracts. Traces of phytochemicals were also seen in less polar solvents like chloroform (steroid and saponin) and benzene (steroid and protein). The nature of extracting solvent plays an important role in extraction of potential compounds of antioxidant activity since the compounds differ in chemical characteristics, polarities and solubilities (Ozarkar 2005). Presence of alkaloids, flavonoids, saponins, terpenoids and phenols were reported in plant extracts of *Camellia sinensis* (purple tea) and the solvents with higher polarity i.e. water, ethanol and acetone were found to extract major phytochemicals groups than non-polar ethyl acetate and chloroform (Geoffrey *et al*. 2014). Methnaol showed better extraction properties than acetone and ethylacetate for extracting few phytochemicals like flavonid, tannin, triterpenes, and lipid and reducing sugar in black packaged tea. Other solvents showed minimum activity (Patil *et al*. 2016). The polar solvents and in some cases even the least polar solvents showed best result in extracting phytochemicals from fresh leaves of TV26 and we could therefore infer that in addition to extraction of samples using solvent with different polarities, extraction time and procedure, the state of sample also matters in phytochemical extraction. The phytochemical constituent slowly degenerates from the time of plucking upto manufacturing. Qualitative screening of phytochemicals is important to the pharmaceutical industry since the presence of a phytochemical of interest may lead to its further isolation, purification and characterization (Ugochukwu and Arukweuche 2013).

Table 1. Qualitative phytochemical screening of TV26 extracted by nine different solvents.

	Hexane	Benzene	Chloroform	Diethylether	Ethylacetate	Acetone	Ethanol	Methanol	Water
Flavonoid	-	-	-	-	++	++++	+	+++	-
Tanin	-	-	-	-	+	++++	++	+++	-
Steroid	-	+	+	++	+++	++++	+++	+++++	+
Terpenoid	-	-	-	-	+	++++	++	+++	-
Cardiac glycoside	-	-	-	-	++	-	++	+++++	+++
Diterpenes	-	-	-	+	++	+++++	+++	++++	-
Coumarin	-	-	-	-	++	++++	++	+++	-
Saponin	-	-	++	-	++	+	+	+++	++
Reducing sugar	-	-	-	+	+	+++	++	++++	-
Protein	-	+	-	+	++	+++	++	++	-

+ Positive test; - negative test; the number of + or - indicates the higher or lower intensity.

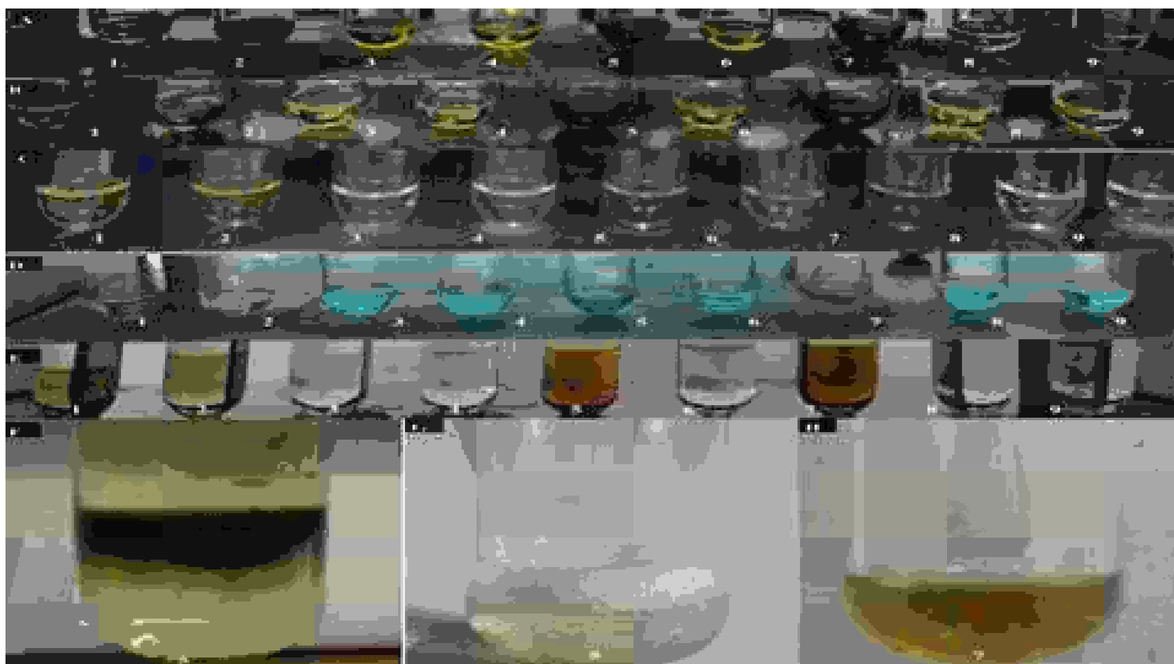

Fig. 1: Qualitative phytochemical screening of TV26 in nine different extracting solvents **1**–Ethanol,**2**–Ethylacetate, **3**-Water, **4**– Benzene, **5**– Methanol, **6**– Diethylether, **7**-Acetone, **8**-Chloroform, **9**-Hexane **[A]** Flavonoid; **[B]** Tanin; **[C]** Coumarin; **[D]** Reducing sugar; **[E]** Terpenoid; **[F]** Cardiac glycoside; **[G]** Saponin; **[H]** Protein.

The antioxidant ability of the sample cannot be concluded only by one method (Patil *et al.* 2016), so we used methods to estimate total phenols quantitatively, DPPH free radical scavenging assay and FRP assay. During DPPH assay, antioxidants acts as a proton donor where the free radical is scavenged and absorbance is decreased thereby rendering change in color from purple to yellow (Liu *et al.* 2010; Manivasagan *et al.* 2015). The highest percent of radical scavenging was recorded in cold water extracts (5mg/ml) i.e. 91.10% and was at par with 93.40% percent scavenging activity of ascorbic acid taken as standard (5mg/ml). Diethylether, ethylacetate, acetone, ethanol, and methanol also gave better results with percent scavenging activity above 50% (Fig. 2). The lowest scavenging activity was seen in hexane extracts. Methanol (Patil *et al.* 2016) ethanol and acetone extract (Turkmen *et al.* 2006b) has already been reported to have strong antioxidant property in black tea. Turkmen (2006) reported varying antioxidant activity with changes in percent concentration of the solvent thus reporting 50% ethanol and 50% acetone with maximum antioxidant potential in mate tea and black tea respectively and also showed the capability of hot water showing moderate antioxidant potential in black tea and higher antioxidant potential in mate tea when compared to other 100% solvent. Thus we could infer that solvent potential may be enhanced or reduced with altering the percent concentration of the solvent.

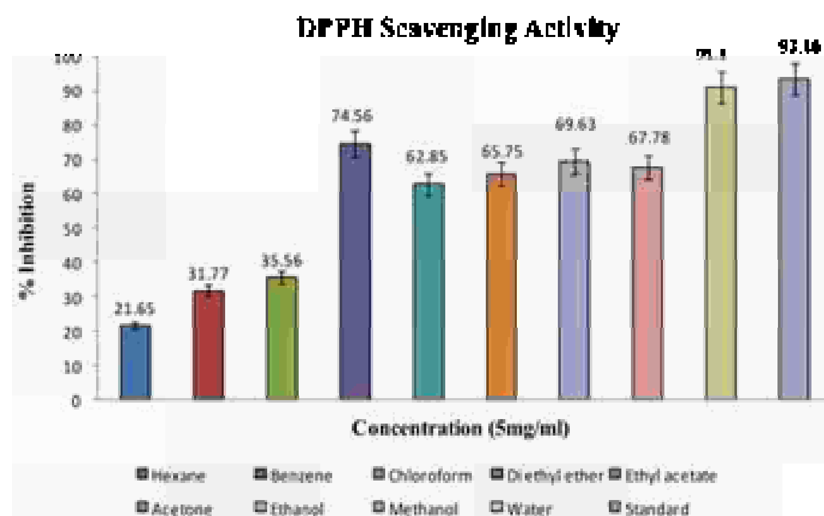

Fig. 2: Percent DPPH free radical scavenging activity of all the nine extracts and ascorbic acid used as standard at concentration 5mg/ml.

The total phenol content was expressed as GAE (mg/g). Methanol, acetone, ethanol and ethyl acetate gave the best results with the total phenol content value recorded as 100.60mg/g, 87.07mg/g, 58.73 mg/g and 51.47mg/g respectively with methanol giving the best result followed by water, diethylether, benzene, chloroform and hexane (Table 2). The lowest value recorded was that of hexane i.e. 4.30mg/g of total phenols. Green tea was found to be a richer source of phenolics than white tea and found 40% aqueous ethanol to be useful for extracting catechin (Rusak *et al*. 2008). Total phenol content estimation following Folin-Ciocalteu reagent method showed 50% acetone, 50% N,N-dimethylformamide (DMF), 50% ethanol and 50% methanol to be suitable for extracting total phenols (Turkmen *et al*. 2006).

Table 2. Determination of total phenolic content (TPC) from fresh leaves of TV26 extracted by different organic solvents.

Solvents	TPC(µg/ml)	GAE (mg/g)	Total phenolic content (mean±SD)
Hexane	2.2	4.3	4.3±0.2
Benzene	3.7	6.9	6.9±0.6
Chloroform	3.0	5.8	5.8±0.2
Diehylether	7.4	12.8	12.8±1.9
Ethylacetate	27.1	51.5	51.5±2.6
Acetone	43.7	87.1	87.1±0.3
Ethanol	31.1	58.7	58.7±3.5
Methanol	54.1	100.6	100.6±7.7
Water	37.5	36.8	36.8±38.2

$y = 0.0075x - 0.0252$; $R^2 = 0.9864$

Acetone extracts showed higher ferric reducing power than other extracts as par with ascorbic acid taken as standard (Fig. 3). The antioxidant compounds acts as reducers causing the reduction of Fe^{3+}/ferricyanide complex to the ferrous form which can be monitored by determining the formation of Perl's Prussian blue at 700nm .The IC_{50} value calculated represents the exact concentration for 0.5 absorbance at 700 nm and the reducing power mostly increases with increasing concentration of antioxidant compounds (Ferreira *et al*. 2007; Manivasagan *et al*. 2015; Patil *et al*. 2016). Acetone extracts showed higher ferric reducing power with IC_{50} value 426.45±1.12 µg/ml compared to the standard (ascorbic acid) 270.35±0.66 µg/ml.

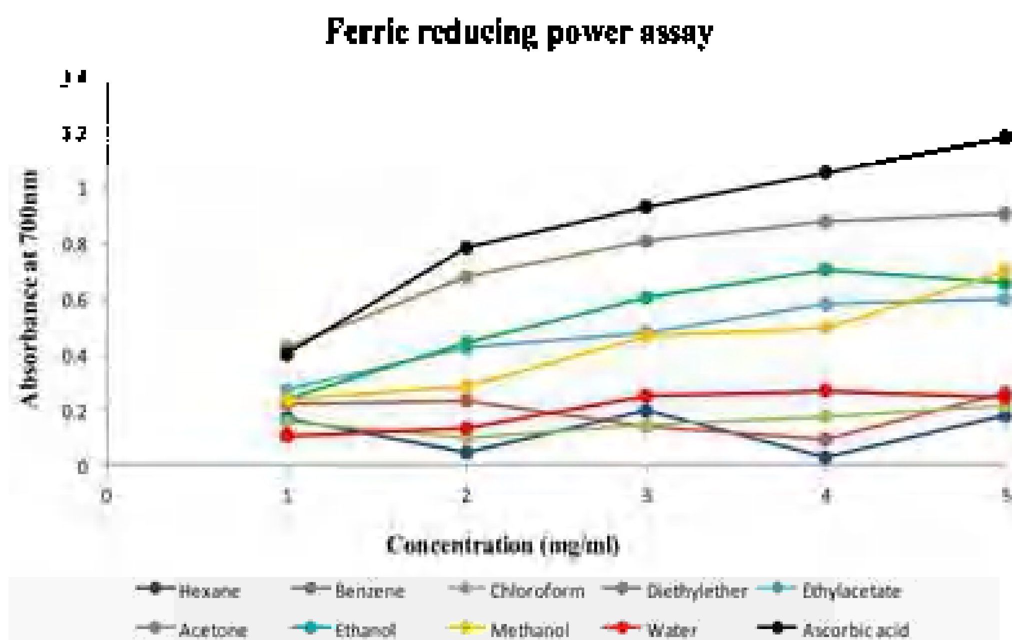

Fig. 3: Ferric reducing power of all the extracts and ascorbic acid (standard) at different concentration (mg/ml).

Conclusion

The qualitative and quantitative phytochemical screening as well as studying the antioxidant activity based on extracting solvents of different polarities was studied for fresh leaves of TV26. The ranges of organic solvent used with differing polarities included absolute hexane, benzene, chloroform diethylether, ethylacetate, acetone, ethanol, methanol and water. Solvents like acetone, methanol, ethanol and ethyl acetate was found to be the most potent solvent for various phytochemical extractions like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin, cardiac glycoside, saponin, protein and reducing sugar. The highest percent of radical scavenging was recorded in cold water extracts (5mg/ml) i.e. 91.10% and was at par with 93.40% percent scavenging activity of ascorbic acid taken as standard (5mg/ml). Methanol, acetone, ethanol and ethyl acetate gave the best results with the total phenol content value recorded as 100.60mg/g, 87.07mg/g, 58.73 mg/g and 51.47mg/g respectively. Acetone extracts showed higher ferric reducing power with IC_{50} value 426.45±1.12 µg/ml compared to the standard 270.35±0.66 µg/ml. The polar solvents and in some cases even the least polar solvents showed best result in extracting phytochemicals from fresh leaves of TV26 and we could therefore infer that

in addition to extraction of samples using solvent with different polarities, extraction time and procedure, the state of sample also matters in phytochemical extraction.

The collective information of this work could promote future research work in broader ways and to check whether the result varies or remain same among other varieties. Elaborate qualitative phytochemical profiling thus gave an idea about the potency of particular solvent in extracting specific compound of interest.

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EFFICACY OF ANTHOCYANIN IN PRODUCTION OF REMEDIAL TEA

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ABSTRACT

Tea is consumed as a medicinal beverage from centuries, as the medicinal component includes polyphenols, caffeine, and amino acids. Apart from that it also contains flavonoids; compounds reported to have antioxidant properties with many beneficial effects. Anthocyanins belonging to the flavonoid group are naturally occurring compounds that imparts colour to fruits, vegetables and plants. Apart from that it has an array of health promoting benefits. This article has been reviewed to highlight the importance of anthocyanin as well as to motivate research in exploring tea varieties with abundant anthocyanin so that consumption of anthocyanin rich tea or beverage and also use of natural dye made using anthocyanin would replace harmful effects of chemical drugs and also improve country's economy by flourishing the tea industry with increased consumption.

Keywords: *Camellia sinensis*, anthocyanin, pharmacological activity, industrial use

INTRODUCTION

Cultivated tea belongs to genus Camellia, consisting of three species each with specific plant types viz. *Camellia sinensis* China type, *Camellia assamica* Assam type and *Camellia assamica lasiocalyx* Cambod type (Wight, 1962). Tea is an important agricultural and commercial crop consumed worldwide, mainly as a beverage made from processed tea leaves and it has also been used for medicinal purposes for several centuries. (Friedman et al., 2007; Wang et al., 2012)According to varying processing procedures, tea currently made in the world can be classified into six main types including black, green, white, yellow, oolong and reprocessed tea (P12). Of all the types, green tea is mostly favored as a medicinal tea as many of its medicinal properties like antioxidative, anti-mutagenic, anticarcinogenic, antihypersensitive, antibacterial, antiviral and also weight reducing property have been already reported (Saito et al., 2011). Besides green tea, purple tea is gaining much importance since, interest in anthocyanins is growing among researchers owing to their potential health benefits(Kong et al., 2003).

Anthocyanins are the another most important plant pigment besides chlorophyll visible to the human eye belonging to the widespread class of phenolic compounds collectively named flavonoids (Kong et al., 2003). They occur in different colour basically red, blue or purple depending upon their pH. Synthesized through the phenylpropanoid pathway, the water soluble vacuolar pigment has many important roles to play besides imparting colour and contributing to astringent sensation.

It is found in many plant species including red grapes (Rivero-Perez et al., 2008), berries (blueberry, strawberry, raspberry, blackcurrant, bil- berry, cranberry, elderberry) (Nicoue et al., 2007), eggplant (Azuma et al., 2008), purple fleshed sweet potatoes (Oki et al., 2002) and flowers like Hibiscus (Lo et al., 2007a). These pigments have been found to be the largest and most important group of water soluble pigments found in nature and they contribute to the attractive colours of fruits, vegetables and flowers imparting red, orange, purple, violet and blue colours (Feild et al., 2001). Anthocyanins in plants normally accumulate in the vacuoles of the epidermal and sub epidermal cells (Steyn et al., 2002). The colours of these pigments are pH-dependent (Mazza and Miniati, 1993). Interest in anthocyanins has recently increased owing to their potential health benefits (Kong, Chia, Goh, Chia, & Brouillard, 2003) and their use as an alternative source of synthetic colourants/dyes. (Jackman et al., 1987; Kerio et al., 2012).

The anthocyanin pigments that create the

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color (Tsai and Ou, 1996) are responsible for the wide range of coloring in many foods. Recently, the biological activities of anthocyanin, such as antioxidant activity, protection from atherosclerosis and anti-carcinogenic activity have been investigated, and shown to have some beneficial effects in the treatment of diseases.

For instance, there have been reports on the antioxidant activity in grape anthocyanin (Igarashi et al., 1989) and the biological effect of anthocyanin in low density lipoprotein and lecithin-liposome systems (Meyer et al., 1997). Anthocyanins were also found to have many times more activity than common antioxidants such as ascorbate (Wang et al., 1997). Overall, there is now increasing evidence that antioxidants in the human diet are of major benefit for health and well-being. (Tsai et al., 2002)

However, no tea has been demonstrated to contain an abundant amount of anthocyanins. Therefore, 'Sunrouge' was developed, a red leaf tea cultivar that is rich in anthocyanins by natural crossing in 2009. An anthocyanin-rich parental line, 'Cha Chuukanbohon Nou 6', which was derived from *C. taliensis x C. sinensis* in 2004, was previously developed. *C. taliensis* is closely related to *C. sinensis*. However, the anthocyanin content of 'Cha Chuukanbohon Nou 6'suddenly diminishes as the leaf matures. Therefore, anthocyanin-rich tea was developed the cultivar which was higher in anthocyanin content than 'Cha chuukanbohon Nou 6', and in which the anthocyanin content did not diminish after leaf maturation. 'Sunrouge' is an offspring of 'Cha Chuukanbohon Nou 6'. Saito *et al*. isolated six anthocyanins from 'Sunrouge' leaves.(Maeda-Yamamoto et al., 2012) Because of the sedentary nature of plants, they are prone to UVirradiation which can cause oxidative stress. Anthocyanins protect plants against such irradiation. Their biosynthesis has been demonstrated to be upregulated when the plant is exposed to UV-B irradiation (Merzlyak et al., 2008). Although anthocyanin is not found abundantly in Camellia sinensis, there are many more purple leaf coloured tea plants that are yet to be explored to make promising anthocyanin rich tea.

BIOCHEMISTRY OF ANTHOCYANIN

Anthocyanin belongs to the widespread class of phenolic compounds collectively named flavonoids. They are glycosides of poly-hydroxy and poly-methoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (Kong et al., 2003). Chemically, anthocyanins are glycoside moieties of anthocyanidins derived from the flavylium (2-phenylbenzopyrilium) cation shown in Fig 1 (Kerio et al., 2012). The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule (Kong et al., 2003).

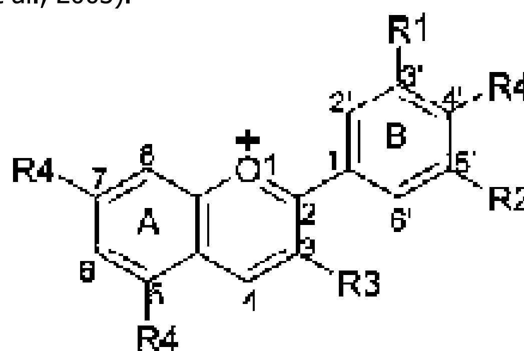

Fig 1: Basic structure of anthocyanidin pigment, the flavylium cation where R1 and R2 are H, OH, or OCH; R3 is a glycosyl or H; and R4 is OH or a glycosyl. (Kong et al., 2003)

There are several anthocyanidins described in nature but among these, six are widespread in fruits and vegetables namely; pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin as shown in Table 1 (Kerio et al., 2012). The various anthocyanidins differs in number and position of the hydroxyl and /or methyl ether groups attached on 3, 5, 6, 7, 3′, 4′ and/or 5′ positions (Table 1). Despite the fact that 31 different monomeric anthocyanidins have been identified (including 3-deoxy-anthocyanidins, pyranoanthocyanidins and sphagnorubins), 90% of the naturally occurring anthocyanins are based on only six structures (30% on cyanidin, 22% on delphinidin,18% on pelargonidin and in summary 20% on peonidin, malvidin and petunidin). Those six anthocyanidins are usually known as common

Table 1: Common anthocyanidins occurring in the nature. *Excerpted from Ananga et al. (2013)*

ANTHOCYANIN COMMON IN HIGHER PLANTS									
Sl. no	Name	Abbreviation	Substitution Pattern						
			3	5	6	7	3´	4´	5´
1	Cyanidin	Cy	OH	OH	H	OH	OH	OH	H
2	Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH
3	Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe
4	Pelargonidin	Pg	OH	OH	H	OH	H	OH	H
5	Peonidin	Pn	OH	OH	H	OH	OMe	OH	H
6	Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH

anthocyanidins (Ananga et al., 2013).The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). The following four classes of anthocyanidin glycosides are common: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides. 3-glycosides occur about two and half times more frequently than 3,5-diglycosides. So, the most widespread anthocyanin is cyanidin 3-glucoside. (Kong et al., 2003)

Because of their polar nature, anthocyanins are soluble in polar solvents, such as methanol (MEOH), ethanol and water. The initial step in their isolation therefore involves solvent extraction, which includes the use of acidified methanol or ethanol. The use of acid stabilizes anthocyanins in the flavylium cation form, which is red at low pH (Mc. Ghie and Walton, 2007).

The color of anthocyanidins differs with the number of hydroxyl groups, attached on their molecules (especially those substituted in ring B). With the increase of attached hydroxyl groups, the visible color of entire molecule shift from orange to violet (Andersen and Jordheim, 2006; Delgado-Vargas et al., 2000; Delgado-Vargas and Paredes-Lopez, 2002; Tanaka et al., 2008).

Glycosylation of anthocyanidins results to additional reddening of obtained anthocyanins, whereas the presence of aliphatic or aromatic acyl moieties causes no color change or slight blue shift and has significant effect on their stability and solubility (Tanaka et al., 2008). Changes in pH can also cause reversible structural transformations in anthocyanins molecules, which has a dramatic effect on their color Delgado-Vargas and Paredes-Lopez, 2002; Wrolstad, 2004). Most of the anthocyanins are *O*-glycosylated at 3 (except those based on 3-deoxyanthocyani-dins and sphagnorubinns), 5 or 7 positions and in some cases at 3´, 4´ and 5´ positions(Ananga et al., 2013). However, 8-*C*-glycosylanthocyanins have been found only in *Tricyrtis formosana* Baker (Saito et al., 2003; Tatsuzawa et al., 2004). Anthocyanins contain two, one or tree monosaccharide units in their molecules. The usual monosaccharide residues are glucose, galactose, arabinose, ramnose, xylose and glucuronic acid. However, anthocyanins containing disaccharides and trisaccharides were also found in nature but no tetrasaccharides have been discovered yet (Ananga et al., 2013).

BIOSYNTHESIS OF ANTHOCYANIN IN TEA

Anthocyanin molecules are produced via flavonoid pathway. Anthocyanin pigments are assembled like all other flavonoids from two

different streams of chemical raw materials in the cell where one stream involves the shikimate pathway to produce the amino acid phenylalanine and the other stream produces three molecules of malonyl-CoA, a C3 unit from a C2 unit (acetyl-CoA). The Shikimate pathway leads to the formation of chorismate, which is the precursor of the aromatic amino acids phenylalanine, tyrosine and tryptophan. Phenylalanine (and in some cases tyrosine, but not in the case of tea plants) is the primary precursor of catechins (Tounekti et al., 2013).The flavonoid pathway starts with phenylalanine, produced via shikimate pathway and trans- formed to 4 coumaroyl-CoA. The key enzyme, chalcone synthase (CHS) produce a naringe-nin chalcone by condensing one molecule of 4-coumaroyl-CoA and three malonyl-CoA molecules (derived from citrate produced by The Krebs cycle). In this case, the rings A and C are derived from the acetate pathway, whereas the ring B is derived from shikimate pathway (Ananga et al., 2013).Currently there are three isoforms of chalcone synthase (Park et al., 2004).The three genes act to synthesize naringenin chalcone, which is used in the formation of anthocyanins, proanthocyanidins, and other phenolic compounds. According to (Ageorges et al., 2006), the three different CHSs may act in three different pathways to produce different secondary metabolites. In the next step, chalcone isomerase (CHI) converts stereospecifically the naringenin chalcone to its isomer naringenin. Ring B of the naringenin undergoes further hydroxylation by the enzymes flavonoid 3´-hydroxylase (F3´H), flavonoid 3´5´-hydroxylase (F3´5´H) or flavanon 3ß-hydroxylase (F3H) (He et al., 2010). Then, the obtained dihydroflavonols are reduced by the enzyme dihydroflavonol 4-reductase (DFR) to the corresponding leucoanthocyanidins. After this reduction, anthocyanidin synthase (ANS) oxidize leucoanthocyanidins to their corresponding anthocyanidins. Anthocyanidins are inherently unstable under physiological conditions and are immediately glycosylated to anthocyanins by UDP-glucose: Anthocianidin: Flavonoid glucosyltransferase (UFGT) (He et al., 2010). Anthocyanins, containing methylated anthocyani-dins (peonidin 4, petunidin 5 and malvidin 6) as aglycone can be obtained by methylation of hydroxyl groups on the ring B of the cyanidin-3-*O*-glucoside 7, delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside by the enzyme *O*-methyltransferase (OMT). Future acylation of produced anthocyanins is possible by the action of different anthocyanin acyltransferases (ACT).

EXTRACTION OF ANTHOCYANIN

Anthocyanins are soluble in polar solvents, and they are normally extracted from plant materials by using methanol that contains small amounts of hydrochloric acid or formic acid. The acid lowers the solution’s pH value and prevents the degradation of the non-acylated anthocyanin pigments. However, as hydrochloric acid or formic acid is concentrated during the evaporation of the methanol-hydrochloric acid or methanol-formic acid solvent, pigment degradation occurs (e.g. in the extract of Azalea cv. Alice Erauw, the cyanidin-3monosides are converted into unstable aglycone). Small amounts of acid may also cause partial or total hydrolysis of the acyl moieties of acylated anthocyanins that are present in some plants. One report compared various techniques for the extraction of anthocyanins from red grapes and demonstrated that solvents containing up to 0.12 mol/l hydrochloric acid can cause partial hydrolysis of acylated anthocyanins (Revilla et al., 1998). Acetone has also been used to extract anthocya nins from several plant sources (GarciaViguera et al., 1998; Giusti et al., 1994). In comparison to acidified methanol, this technique allows an efficient and more reproducible extraction, avoids problems with pectins, and permits a much lower temperature for sample concentration (GarciaViguera et al., 1998). Solid-phase extraction (SPE) on C_{18} (SPE) cartridges or Sephadex is commonly used for the initial purification of the crude anthocyanin extracts. The anthocyanins are bound strongly to these adsorbents through their unsubstituted hydroxyl groups and are separated from unrelated compounds by using a series of solvents of increasing polarity. Sunrouge’ tea leaves i.e., anthocyanin rich tea leaves (87.2 g) were extracted with 15% acetic acid (600 mL 3), and additionally extracted with 15% acetic acid-

containing 50% EtOH (600 mL 4) (Saito et al., 2011). The anthocyanins were extracted successfully from tea products processed from a number of newly bred purple leaf coloured Kenyan tea cultivars (*Camellia sinensis*) using acidified methanol/HCl (99:1 v/v). (Kerio et al., 2012).

CHARACTERIZATION OF ANTHOCYANIN

The characterization of a mixture of anthocyanins usually involves the separation and collection of each compound, and subsequent analysis by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectroscopy (FAB-MS). For the separation and structural analysis, the use of liquid chromatography–mass spectrometry (LC–MS) technique, which combines the separation of LC with the selectivity and sensitivity of the MS detector, permits the identification of individual compounds in a mixture of compounds. Recently liquid chromatography-electron impact ionization mass spectrometry (LC-EI-MS) was also used to identify the anthocyanins of Catharanthus roseus extracts (Piovan et al., 1998). LC–MS with an atmospheric pressure-ionization ion-spray interface to analyze the anthocyanins contained in the grape skins (Vitis vinifera L.). Nineteen derivatives of cyanidin, delphinidin, petunidin, malvidin and peonidin were identified by this ionization technique. The individual mass spectra showed peaks for the molecular ions, together with a fragment corresponding to aglycone; when acylation was present, an additional fragment was detected at mass/charge values corresponding to the loss of acyl moiety from the molecular ion (Baldi et al., 1995). Many new acylated anthocyanins have been found with the help FAB-MS (Saito et al., 1983).

Atmospheric-pressure ionization (API) techniques have several advantages over other MS detection methods. In API-MS the ion source is located outside the MS; the ions are formed at atmospheric pressure, and then sampled into the mass spectrometer. These are soft ionization techniques (only the molecular ion is formed), although the application of a potential at the entrance of the mass spectrometer (fragment voltage) creates suitable conditions for CID, and the production of fragment ions. Two API interfaces are available commercially, namely, the atmospheric pressure chemical ionization interface (APCI) and the ESI interface. LC–MS system equipped with an ESI interface was used to analyze anhocyanins present in extracts of grape skins and red wine (Revilla et al., 1999) Another technique recently used for anthocyanin analysis is capillary electrophoresis (CE) which has excellent mass sensitivity, high resolution, low sample consumption and minimal generation of solvent waste. The separation of a mixture of standards, as well as strawberry and elderberry anthocyanins, by capillary zone electrophoresis (CZE) has already been reported by Bridle et al. (1997). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS) to perform both qualitative and quantitative analyses of anthocyanins in wine and fruit juice, and matrix-assisted laser desorption/ionization time-offlight mass spectrometry (MALDI–TOF–MS) to analyze the content of anthocyanins in various foods (Wang and Sporns, 1999; Wang and Lin, 2000). Purification and isolation of 6 anthocyanins from 'Sunrouge' by chromatography, and identification of them by LC/MS/MS and NMR analysis has already been reported.(Saito et al., 2011).

NMR, an important technique is useful for structural elucidation of anthocyanin. Some examples of its use are given where it has been reported that the separation of anthocyanins from red radish (Raphanus sativus) and their structural elucidation by one- and two-dimensional NMR.

Four anthocyanins were obtained: pelargonidin 3-O- [2-O-(b-glucopyranosyl) -6-O-(trans-p-coumaroyl) -b-glucopyranoside] 5O-(6-O-malonyl-b-glucopyranoside); pelargonidin 3-O[2-O -(b-glucopyranosyl)-6-O- (trans-feruloyl)-b-glucopyranoside] 5-O-(6-O- malonyl-b-glucopyranoside); pelargonidin 3-O-[2-O-(b-glucopyranosyl)-6-O- (trans-pcoumaroyl)-b-d-glucopyranoside] 5-O-(b-glucopyranoside) and pelargonidin 3-O-[2-O-(b-glucopyranosyl)-6O-(trans-feruloyl)-b- gluco-pyranoside] 5-O-(b-glucopyr-anoside). They also investigated the three-dimensional conformation of the molecule by using NOESY techniques, which showed

proximity between the hydrogen from the cinnamic acid acylating group and the C-4 of the pelargonidin (Giusti et al., 1998). Similarly, anthocyanin trisaccharides of *Vaccinium padifolium* were identified by using NMR and other techniques (Cabrita et al., 2000). Anthocyanins from Kenyan teas were purified by C_{18} solid phase extraction (SPE) catridges and characterised by HPLC-UV–Visible. They were identified according to their HPLC retention times, elution order and comparison with authentic standards that were available. Total monomeric anthocyanins were determined by the pH-differential method.(Kerio et al., 2012). Anthocyanins content in purple tea was measured by single-pH and the content was much more than that measured by pH-Differential spectrophotometry. The two methods showed good linear relation(R2=0.9927).The effects of interfering substances on quantitative analysis of anthocyanins could be eliminated by using pH-Differential spectrophotometry. The results provided a reliable basis for the measurement of Anthocyanins (Chen and Lu, 2011).

ANTHOCYANIN RICH TEA

Sunrouge is the red tea cultivar made by naturally crossing *Camellia taliensis* and *Camellia sinensis* for which an application for registration was made in 2009. An anthocyanin-rich parental line, 'Cha Chuukanbohon Nou 6', was previously developed by crossing *C. taliensis x C. sinensis* in 2004 where *C. taliensis* is closely related to *C. sinensis*. However, the anthocyanin content of 'Cha Chuukanbohon Nou 6 suddenly diminished after maturation of leaves. Therefore Sunrouge was introduced as an offspring of 'Cha Chuukanbohon Nou 6' whose anthocyanin content did not diminish after leaf maturation (Maeda-Yamamoto et al., 2012).The total anthocyanin content of four tea cultivars: 'Sunrouge', 'Cha Chuukanbohon Nou 6', 'Benibana-cha' (C. sinensis), the anthocyanin-rich tea cultivar, and 'Yabukita' (C. sinensis), the common tea cultivar in Japan was quantified and it has been already reported that the anthocyanin content of 'Sunrouge' was the highest among 4 tea cultivars, and was 8.4 times higher than that of 'Yabukita'. Purification and isolation of 6 anthocyanins from 'Sunrouge' has already been done using chromatography, and also has been identified using LC/MS/MS and NMR analysis, where the four anthocyanins were identified as delphinidin-3-O- ßD-(6-(E)-pcoumaroyl) galactopyranoside(2),delphinidin-3-O-ßD-(6-(E)-p-coumaroyl) glucopyranoside (3), cyanidin-3-O-ß-D-(6-(E)-pcoumaroyl) galacto-pyranoside (4), and cyanidin-3-O-ßD-(6-(E)-p-coumaroyl)glucopyranoside(5), and the other two were estimated respectively as delphinidin-(Z)-p-coumaroylgalactopyranoside (1), petunidin-(E)-p-coumaroylgalactopyranoside (6). Compound 3 was found in tea for the first time. In general, anthocyanins has been reported to have various bioactivities, including relieving eyestrain and antioxidative effects, so it is expected that drinking 'Sunrouge' tea would bring in the above bioactivities.(Saito et al., 2011).

Another anthocyanin rich tea was extracted from tea products processed from a number of newly bred purple leaf coloured Kenyan tea cultivars (Camellia sinensis) using acidified methanol/HCl (99:1 v/v). Extracted anthocyanins were purified by C_{18} solid phase extraction (SPE) catridges and characterised by HPLC-UV–Visible. Of the six most common natural anthocyanidins, five were identified in the purified extracts from purple leaf coloured tea, in both aerated (black) and unaerated (green) teas namely; delphinidin, cyanidin, pelargonidin, peonidin and malvidin. The most predominant anthocyanidin was malvidin in both tea products. In addition, two anthocyanins namely, cyanidin-3-O-galactoside and cyanidin-3-O-glucoside were also identified. (Kerio et al., 2012).

ANTHOCYANIN AND ITS UTILITY IN PLANTS

Apart from imparting colour to the plants, anthocyanin play a definite role in the attraction of animals for pollination and seed dispersal, and hence they are of considerable value in the co-evolution of these plant-animal interactions. Anthocyanins and 3-deoxyanthocyanidins however have roles in flowering plants other than as attractants. They can act as antioxidants, phytoalexins or as antibacterial

agents. Anthocyanins may be important factors along with other flavonoids in the resistance of plants to insect attack(Harborne, 1988). For example, cyanidin 3-glucoside was shown to protect cotton leaves against the tobacco budworm (Hedin and Hedin, 1983).. In photosynthetic tissues (such as leaves and sometimes stems), anthocyanins have been shown to act as a "sunscreen", protecting cells from high-light damage by absorbing blue-green and ultraviolet light, thereby protecting the tissues from photo-inhibition, or high-light stress. This has been shown to occur in red juvenile leaves, autumn leaves, and broad-leaf evergreen leaves that turn red during the winter. The red coloration of leaves has been proposed to possibly camouflage leaves from herbivores blind to red wavelengths, or signal unpalatability, since anthocyanin synthesis often coincides with synthesis of unpalatable phenolic compounds (Sullivan, 1998). Some roles of anthocyanin have been tabulated in Table 2.

PHARMACOLOGICAL ACTIVITY

Anthocyanins possess known pharmacological properties and are used by humans for therapeutic purposes. Following the recognition that pigment extracts are more effective than O-(b-hydroxyethyl) rutin in decreasing capillary permeability and fragility and in their anti-inflammatory and anti-oedema activitiest is possible that anthocyanins may replace rutin and its derivatives in the treatment of illnesses involving tissue inflammation or capillary fragility. The crude anthocyanin extracts of *Vaccinium myrtillus* have been given orally and by intravenal or intramuscular injection to reduce capillary permeability and fragility. (Kong et al., 2003)

Anthocyanins were not found effective in suppressing tumor growth (Ghiselli et al., 1998). However, an antioxidant activity study of anthocyanin fractions from Italian red wine showed that the anthocyanin fraction was the most effective both in scavenging reactive oxygen species and in inhibiting lipoprotein oxidation and platelet aggregation (Ghiselli et al., 1998). This result suggests that anthocyanins could be the key component in red wine that protects against cardiovascular disease. Another report on the anti-tumor activity of anthocyanins was reported where they found that the anthocyanin fraction from red wine suppressed the growth of HCT-15 cells, which are derived from human colon cancer or

Table 2: The role of anthocyanins and 3-deoxyanthocyanidins in plants. *Excerpted from Kong et al. (2003)*

Plant	Compound	Origin	Function
	Angiosperms		
Senecio cruentus	Cinerarin	Petals	Pollination
Sorghum	Apigeninidin	Leaf sheath	Phytoalexin anti-microbial antioxidants
	Gymnosperms		
Abies concolor	Petunidin-3- glucoside Cyanidin-3- glucoside	Cone	-
Pinus contorta	Anthocyanin	Leaves	Cold tolerance
Pinus banksiana	-	Seedlings	Photoinhibition tolerance
	Ferns		
Davallia divaricata	Pelargonidin-3-p-coumaryl-glc-5-glc (monardein)	Young leaves	-
Ferns species	Apigenidin	Leaves	-
	Mosses		
Bryum, Splachunm	Luteolinidin-5-glc	Leaves	-
	Liverwort		
Cephaloziella exilifolia	Anthocyanin like	Thallus	-

AGS cells from human gastric cancer. The suppression rate by the anthocyanin fraction was significantly higher than that of the other fractions (Kamei et al., 1998). The ability of anthocyanin obtained from the petals of *H. rosa-sinensis* was examined which prevented carbon tetrachloride-induced acute liver damage in rats. The results showed that those rats treated with anthocyanin and carbon tetrachloride had significantly less hepatotoxicity ($P<0.05$) than those given carbon tetrachloride alone. This was assessed by measuring the levels of serum aspartate and alanine aminotransferase activities 18 hours after carbon tetrachloride was given. This result suggested that *H. rosa-sinensis* anthocyanin may be protective against carbon tetrachloride-induced liver injury (Obi et al., 1998).

The antimutagenicity of water extracts prepared from the storage roots of four varieties of sweet potato with different flesh colors, using Salmonella typhimurium TA 98. They found that two anthocyanin pigments purified from the purple colored sweet potato 3-(6,6´-caffeylferulylsophoroside) 5-glucoside of cyanidin (YGM-3) and peonidin (YGM6), effectively inhibited the reverse mutation induced by heterocyclic amines-mutagen, Trp-P-1, Trp-P-2, and IQ in the presence of rat liver microsomal activation systems (Yoshimoto et al., 1999).

It has been reported that the the administration of anthocyanin dyes from Aronia melanocarpa to rats before the intraperitoneal injections of PlateletActivating Factor (PAF) and ceruleine had a beneficial effect on the development of acute experimental pancreatitis in rats (Jankowski et al., 2000). It was revealed that this was due to the reduction of pancreatic swelling and a decrease in lipid peroxidation and adenosine deaminase activity.

They also examined the effect of anthocyanins from Cabernet red wine on the course and intensity of symptoms of experimental diabetes in rats (Jankowski et al., 1999). The results showed that a simultaneous daily administration of anthocyanins obtained from Cabernet red wine and streptozotocin substantially decreased sugar concentrations in the urine and blood serum. These anthocyanins also inhibited the loss of body mass caused by the injection of streptozotocin. Simultaneously, the anthocyanin pigment prevented the generation of free oxygen radicals, and decreased the peroxidation of lipids. The influence of anthocyanins was determined from chokeberries on the generation of autoantibodies to oxidize low density lipoproteins (oLAB) in pregnancies complicated by intrauterine growth retardation (IUGR). An experiment was conducted with a study group of 105 pregnant women (on the turn of trimester two according to LMP) with IUGR (sonographic examination results below the 5th percentile for real gestational age) who were randomly divided into 2 groups. Fifty women were administered anthocyanins and 55 women were given a placebo. There was a control group of 60 healthy pregnant women. They then examined the level of oxidative stress measured by the serum concentration of autoantibodies required to oxidize low density lipoproteins (oLAB). In the anthocyanin group, the oLAB titres decreased from 1104_41 mU/ml before treatment to 752_36 mU/ml in the first month and 726_35 mU/ml in the second month, at $P<0.01$. In the placebo group, the oLAB titres showed a slightly increasing trend: 1089_37 mU/ml before treatment, 1092_42 mU/ml in the first month and 1115_43 mU/ml in the second month, at $P>0.05$. The oLAB titres in the control group were 601_49 mU/ml before treatment, 606_45 mU/ml in the first month, and 614_43 mU/ml in the second month, at $P>0.05$. The results indicated that natural antioxidants (anthocyanins) can be useful in controlling oxidative stress during pregnancies complicated by IUGR (Pawlowicz et al., 2000).

Hibiscus anthocyanins (HAs), a group of natural pigments occurring in the dried flowers of *Hibiscus sabdariffa L*., are used in soft drinks and herbal medicines. Their antioxidant bioactivity has been studied and it appears that HAs can significantly decrease the leakage of lactate dehydrogenase and the formation of malondialdehyde induced by a treatment of tert-butyl hydroperoxide (t-BHP). The in vivo investigation showed that the oral pretreatment of HAs before a single dose of t-BHP significantly lowered the serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) and reduced oxidative liver

damage. The histopathological evaluation of the liver revealed that hibiscus pigments reduce the incidence of liver lesions including inflammation, leucocyte infiltration, and necrosis induced by t-BHP in rats (Wang et al., 2000).

Their pharmaceutical value has been additionally increased due to their high bioavailability. However, the administration and metabolism of Anthocyanins *in vivo* have been investigated in details mostly in rats, whereas the detailed studies on humans still are scantly presented in scientific literature (He and Giusti, 2010; Yue et al., 2011).

The colorful anthocyanins are the most recognized, visible members of the bioflavonoid phytochemicals. The free-radical scavenging and antioxidant capacities of anthocyanin pigments are the most highly publicized of the modus operandi used by these pigments to intervene with human therapeutic targets, but, in fact, research clearly suggests that other mechanisms of action are also responsible for observed health benefits (Lila, 2004). Anthocyanin isolates and anthocyanin-rich mixtures of bioflavonoids may provide protection from DNA cleavage, estrogenic activity (altering development of hormone-dependent disease symptoms), enzyme inhibition, boosting production of cytokines (thus regulating immune responses), anti-inflammatory activity, lipid peroxidation, decreasing capillary permeability and fragility, and membrane strengthening (Lila, 2004).

The roles of anthocyanin pigments as medicinal agents have been well-accepted dogma in folk medicine throughout the world, and, in fact, these pigments are linked to an amazingly broad-based range of health benefits. For example, anthocyanins from *Hibiscus sp* have historically been used in remedies for liver disfunction and hypertension; and bilberry (*Vaccinium*) anthocyanins have an anecdotal history of use for vision disorders, microbial infections, diarrhea, and diverse other health disorders (Rice-Evans and Packer, 2003; Smith et al., 2000; Wang et al., 2000).

But while the use of anthocyanins for therapeutic purposes has long been supported by both anecdotal and epidemiological evidence, it is only in recent years that some of the specific, measurable pharmacological properties of isolated anthocyanin pigments have been conclusively verified by rigorously controlled in vitro, in vivo, or clinical research trials (Tsuda et al., 2003). For example, visual acuity can be markedly improved through administration of anthocyanin pigments to animal and human subjects, and the role of these pigments in enhancing night vision or overall vision has been particularly well documented (Matsumoto et al., 2001). Oral intake of anthocyanosides from black currants resulted in significantly improved night vision adaptation in human subjects (Nakaishi et al., 2000) and similar benefits were gained after administration of anthocyanins from bilberries (Muth et al., 2000). Three anthocyanins from black currant stimulated regeneration of rhodopsin (a G-protein-coupled receptor localized in the retina of the eye), and formation of a regeneration intermediate was accelerated by cyanidin 3-rutinoside (Matsumoto et al., 2003). These studies strongly suggest that enhancement of rhodopsin regeneration is at least one mechanism by which anthocyanins enhance visual acuity. In both in vitro and in vivo research trials, anthocyanins have demonstrated marked ability to reduce cancer cell proliferation and to inhibit tumor formation (Lila, 2004). Anthocyanins inhibit tumorigenesis by blocking activation of a mitogen-activated protein kinase pathway. This report provided the first indication of a molecular basis for why anthocyanins demonstrate anticarcinogenic properties.

The role of anthocyanins in cardiovascular disease protection is strongly linked to oxidative stress protection. Since endothelial dysfunction is involved in initiation and development of vascular disease, four anthocyanins isolated from elderberries were incorporated into the plasma. Crude anthocyanin extracts from bilberry have been administered both orally and via injection to reduce capillary permeability (Kong et al., 2003). Protection from heart attacks through administration of grape juice or wine was strongly tied to the ability of the anthocyaninrich products to reduce inflammation and enhance capillary strength and permeability, and to inhibit platelet formation and enhance nitric oxide (NO) release (Folts, 1998).

Their important function in cognitive decline and neural dysfunction has been investigated and found that fruit extracts (from blueberry)

including anthocyanins were effective in reversing age-related deficits in several neural and behavioral parameters, e.g. oxotremorine enhancement of a K1 evoked release of dopamine from striatal slices, carbachol-stimulated GTPase activity, striatal Ca buffering in striatal synaptosomes, motor behavioral performance on the rod walking and accelerod tasks, and Morris water maze performance and thus proved to improve neural and behavioral parameters (memory and motor functions. (Joseph et al., 1999).

It has already been reported that the Anthocyanins extracted from purple corn, when provided to mice in tandem with a high-fat diet, effectively inhibited both body weight and adipose tissue increases. Typical symptoms of hyperglycemia, hyperinsulinemia, and hyperleptinemia provoked by a high-fat diet did not occur when mice also ingested isolated anthocyanins. The experiments suggest that anthocyanins, as a functional food component, can aid in the prevention of obesity and diabetes(Tsuda et al., 2003).

MEDICINAL BENEFITS OF ANTHOCYANIN RICH FOODS

Consumption of anthocyanin adds as a beneficiary source of neutraceuticals and therapeutics and it has various application in the pharmaceutical industry. Recurrent consumption of anthocyanins could provide various health benefits including reduced risk of coronary heart diseases, anti-carcinogenic activity, antioxidant activity, reduced risk of stroke, anti-inflammatory effects etc. (Davies, 2009; Lila, 2004; Stintzing and Carle, 2004; Wrolstad, 2004).

Biological activity of anthocyanin has already been discussed in the above section. Anthocyanin in diet inhibits body weight and adipose tissue increase that could prevent symptoms of hyperglycemia, hyperinsulinemia, and hyperleptinemia provoked by a high-fat diet and thus can aid in the prevention of diabetes and obesity (Tsuda et al., 2003). It improves and cures vision disorders, microbial infections, diarrhea, and diverse other health disorders (Rice-Evans and Packer, 2003; Smith et al., 2000; Wang et al., 2000). It has the capacity to modulate cognitive and motor function, to enhance memory, and to have a role in preventing age-related declines in neural function (Lila 2004). It also reduces inflammation and capillary fragility (Kong et al., 2003), scavenges reactive oxygen species and in inhibiting lipoprotein oxidation and platelet aggregation (Ghiselli et al., 1998), provides protection from DNA cleavage, estrogenic activity (altering development of hormone-dependent disease symptoms), enzyme inhibition, boosting production of cytokines (thus regulating immune responses).

Above all anthocyanin has many other health beneficial properties, which include antioxidant (Bae and Suh, 2007), anticarcinogenic (Lee et al., 2009), anti-angiogenic (Bagchi et al., 2004), antimicrobial (Viskelis et al., 2009) antiapoptotic (Elisia and Kitts, 2008) and pro-apoptotic (Lo et al., 2007b) properties. Despite their health enhancing properties, no work had been carried out to determine the presence of these coloured polyphenols in newly bred purple leaf coloured tea clone (Kerio et al., 2012).

UTILITY IN VARIOUS INDUSTRIES

The world market of natural food colorants expands with the annual growth rate of 4-6% (Cormier et al., 1996). In USA 4 of the 26 colorants approved by the food administration, that are exempt from certification, are based on anthocyanin pigments (Wrolstad, 2004). In European Union, all anthocyanin-containing colorants are classified as natural colorants under the classification E163 (Socaciu, 2007).

Currently most of the worldwide anthocyanins supply comes from processing of grape pomace, which is a waste product from wine making. But in European Union other plant sources such as red cabbage, elderberry, black currant, purple carrot, sweet potato, and red radish are also allowed (Mortensen, 2006). Anthocyanins, produced by grape cell suspensions can be a promising alternative supply of natural colorants. It has already been demonstrated that the produced pigments by the grape cell suspensions undergo significant structural modifications. Grape cell suspensions accumulates higher levels of metabolically more

evolved structures (methylated and acylated anthocyanins). Acylated anthocyanins are suitable for application in food products, mainly because of the improved color stability compared to non-acylated structures (B¹kowska-Barczak, 2005). Moreover, the grape cell suspensions can also produce elevated levels of beneficial phenolic compounds such as flavonoids, stilbenes, phenolics, etc., which are capable of increasing the added value of the final additive. The overall metabolite profile of grape cells in combination with the lack of microbial and toxic contaminations will give the potential for development of new types of food additives if the entire cell suspension biomass are utilized.

The commercial interest of cosmetic companies to apply plant additives, derived by biotechno-logical cultivation of plant cells to their products has increased remarkably in the last few years (Schurch et al., 2008). The addition of plant cell derived extracts in cosmetic products has been considered as a powerful approach used to increase their health benefits. Several plant extracts have been added to various cosmetic products as moisturizers, antioxidants, whitening agents, colorants, sunscreens, preservatives. With the advancement of plant cell biotechnology, more and more cosmetic companies have been attracted for application of additives, based on plant cell suspensions. Recently the application of so-called plant "steam" cells attracts industry's attention (Schurch et al., 2008). In the last few years, the French company "Sederma" launched the product "Re- sistem™" based on application of *in vitro* cultivated plant cells (www.sederma.fr). The other company, "Mibelle Biochemistry", situated in Switzerland, developed a "PhytoCellTec" prod- uct, based on grape cell suspension of *V. vinifera* L. *cv.* Gamay Fréaux, which was processed by high-pressure homogenizer to produce liposomes for application in cream products (www.mi-bellebiochemistry.com). According to the company, the grape cell derived liposomes contained higher amounts of anthocyanins and when applied on skins serve as strong UV protectors and fight photoaging. The presented examples clearly demonstrate the commercial interest to application of grape cell suspension derived products. However, it is a matter of time for the scientists to develop the biotechnological approach of producing anthocyanins by grape cell suspensions from the frame of experimental scale to commercially applicable products.

Anthocyanins have also been employed to produce juices and red wine whose natural colour as well as high antioxidant property adds to the quality of product.

Anthocyanins can also be used as pH indicators because their color changes with pH; they are pink in acidic solutions (pH < 7), purple in neutral solutions (pH ~ 7), greenish-yellow in alkaline solutions (pH > 7), and colourless in very alkaline solutions, where the pigment is completely reduced (Michaelis et al., 1936) and thus, it is employed in many chemical or pharmaceutical industry as well as in the fied of research.

Nowadays anthocyanins are being used widely in organic solar cells because of their ability to convert light energy into electrical energy (Cherepy et al., 1997). he many benefits to using dye-sensitized solar cells instead of traditional pn junction silicon cells include lower purity requirements and abundance of component materials, such as titania, as well as the fact they can be produced on flexible substrates, making them amenable to roll-to-roll printing processes (Gratzel, 2003).

CONCLUSION AND FUTURE PROSPECTS

Anthocyanins represent a class of important antioxidants, as they are so common in human foods. In recent years, many papers have been published on the in vitro antioxidant activity of anthocyanins and their other functions. However, there are still fewer studies on anthocyanin compared to the studies of other flavonoids. On the other hand tea is a pleasant, popular, socially accepted, economical and safe drink that is initially taken as medicine and later as beverage and now, it has proven well as future potential of becoming an important industrial and pharmaceutical raw material. As green tea, the purple coloured anthocyanin rich tea may also be a popular health drink since anthocyanins has many medicinal properties and is particularly known to be beneficial against cardiovascular diseases, for providing anticancer

benefits, improving vision, cholesterol and blood sugar metabolism as discussed above in the article. Most importantly it sports much lower caffeine content than black or green tea which is beneficial in beverage. Anthocyanin content in tea in addition to other polyphenols and other medicinal compounds would add a splendid color as well as an enigmatic healing property.

Based on these facts, this review is directed to highlight the importance of anthocyanins in order to improve further research in this field, discovering tea cultivars or wild tea plant, rich in anthocyanin.

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Symbiosis
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Comparative genomics of *Prauserella* sp. Am3, an actinobacterium isolated from root nodules of *Alnus nepalensis* in India

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Abstract A novel actinomycete strain, assigned as Am3, was isolated from the root nodules of *Alnus nepalensis* at Mirik hills, India. Analysis of the 16s rRNA gene sequence placed this new strain within the genus *Prauserella.* The genome was sequenced by Illumina sequencing and resulting 5.33-Mbp high quality draft genome sequenced with a G + C content of 70.0 % and 4828 candidate protein-encoding genes. Phylogenetically, *Prauserella* clusters very close to *Amycolatopsis* and was previously placed under the genus *Amycolatopsis*. Our main focus was to reveal the genomic similarities and dissimilarities of the newly sequenced *Prauserella* sp. Am3 with the type strain, *Prauserella rugosa* DSM 43194 T, and to determine its relationship with *Amycolatopsis*, which is happened to be the closest genus of *Prauserella*. Taking an in silico approach, bioinformatic analysis revealed that the core genome of *Amycolatopsis* and *Prauserella* contained 1589 genes. The two *Prauserella* genomes shared approximately 4224 genes, and 237 and 245 unique genes were found in the *P. rugosa* and *Prauserella* sp. Am3 genomes, respectively. Analysis of various phylogenetic trees including a 16s rRNA gene tree, MLSA protein-based tree and concatenated core-genome-based tree, placed both *Prauserella* genomes together with *Amycolatopsis halophila* YIM 93233 as its closest neighbor. Blast Matrix analysis of the predicted proteomes revealed about 86 % homology between the two *Prauserella* genomes. Analysis of the strand variation property revealed the absence of replication-transcriptional selection. Overall, a high degree of similarity was found between the two *Prauserella* genomes and a high percentage of similarity occurred among the *Prauserella* genomes and *Amycolatopsis halophila*.

Keywords Sequencing · MLSA · ANI score · Genome plasticity · Pan-core plot

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1 Introduction

Actinorhizal plants are a group of dicotyledonous plants that form a symbiotic association with the nitrogen-fixing actinobacterium *Frankia* which results in root nodule structures. These plants are distributed among eight orders, eight families, 24 genera and 194 species of angiosperms (Benson and Silvester 1993; Normand and Fernandez 2009). Actinorhizal plants are found worldwide and in diverse environments ranging from arctic tundra (*Dryas* spp.) and alpine forest (*Alnus* sp., *Coriaria* sp. etc) to coastal and xeric condition (*Casuarina* sp.). *Frankia* in symbiosis with actinorhizal plants fixed sustainable amount of molecular nitrogen (ranges from 22 to 300 kg of N_2 per ha per year) and are comparable to those of leguminous plants (Hibbs and Cromack 1990; Wall 2000; Wheeler and Miller 1990). As early visitors of marginal soils, actinorhizal plants are considered pioneer species in the landslides and other threatened areas.

Beside *Frankia*, different actinobacteria genera have also been isolated from the rhizosphere and root nodules of actinorhizal plants (Ghodhbane-Gtari and Tisa 2014; Ghodhbane-Gtari et al. 2010; Liu et al. 2009; Carro et al. 2013; Trujillo et al. 2006; Valdes et al. 2005). Many of these diverse actinobacteria have been characterized up to the genus level and include *Streptomyces*, *Nocardia*, *Micromonospora*, and *Actinoplanes*. These non-*Frankia* actinobacteria are filamentous, and mostly fall into the Actinomycetales category. Colonies and cultures of the non-*Frankia* isolates showed a wide range of colors and physiological traits. These pigmentations ranged in color from white, orange, yellow to maroon (Ghodhbane-Gtari and Tisa 2014), pink to brownish red (Liu et al. 2009), intense orange (Trujillo et al. 2006), and yellow-white (Carro et al. 2013; Valdes et al. 2005). Many of them produce highly branched filaments with some exceptions including *Nocardia* and *Micromonospora* (Maldonado and Quintana 2015).

An isolate from cattle rumen was first designated as *Nocardia rugosa* by Di Marco and Spalla (1957) and latter classified as *Amycolatopsis rugosa* (Lechevalier et al. 1986). However, *A. rugosa* differed in physiological and chemical properties from many of the known *Amycolatopsis* strains and also clustered differently in 16s rDNA sequence based phylogenetic tree (i.e. *A. rugosa* clustered with the *Sacharomonospora* genus instead of being grouped together with other *Amycolatopsis* strains) (Kim and Goodfellow 1999). Based on these physiological and genotypical differences, a new genus *Prauserella* gen nov. was proposed and *Amycolatopsis rugosa* was renamed as *Prauserella rugosa* (Kim and Goodfellow 1999). Since then, many different species have been identified including *Prauserella halophila* (Li et al. 2003), *Prauserella alba* (Li et al. 2003), *Prauserella salsuginis* (Li et al. 2009), *Prauserella flava* (Li et al. 2009), *Prauserella aidingensis* (Li et al. 2009), *Prauserella sediminis* (Li et al. 2009), *Prauserella manina* (Wang et al. 2010) and *Prauserella muralis* (Schafer et al. 2010; Solanki and Kothari 2011). Members of this genus are aerobic, Gram-positive, non-acid-alcohol-fast and non-motile filamentous actinomycetes (Kim and Goodfellow 1999). These actinobacteria produce branched substrate mycelium having a diameter of 0.6–0.8 μm. After 24–48 h growth on rich medium, the hyphae fragment into irregular rods. One of the major characteristics of *Prauserella* is the absence of aerial hyphae. The temperature range for their growth is 10–45 °C with optimum temperature of 34 °C and the optimum pH range is 6.8–7.2 (Kim and Goodfellow 1999).

During the process of isolating *Frankia* from root nodules of *Alnus nepalensis*, an actinobacterium was isolated and 16s rRNA gene analysis identified it as a member of the genus *Prauserella.* Although physiological studies are available on this genus, *Prauserella rugosa* DSM 43194 T, the type strain of this genus, is the only genome (Genbank Accession Number- NR_024889) from this genus that has been sequenced. In this study, we have taken a genomic approach and have sequenced the genome of this new *Prauserella* strain Am3 isolated from the root nodules of *Alnus nepalensis.* The genome of *Prauserella* strain Am3 was analyzed and compared to the *P. rugosa* DSM 43194 T and some selected *Amycolatopsis* genomes, which are very close to *Prauserella* genus.

2 Materials and methods

2.1 Isolation procedures

Nodules from *Alnus nepalensis* were collected along the Siliguri –Darjeeling road via Mirik-Pasupati-Ghoom-Sonada (26.8870° N, 88.1870° E) of West Bengal, India. As a road side tree, *A. nepalensis* is commonly found in abundance at this location. Fresh, young and light-brown-colored root nodules from young *A. nepalensis* were collected in the Mirik hills during the summer 2007. The nodule lobes were surface sterilized with a series of mild surface-sterilizing agents as previously described (Bose and Sen 2006). The nodule lobes were incubated in Qmod medium (Lalonde and Calvert 1979) supplemented with 3 % activated charcoal for 48 h at 30 °C. Activated charcoal was used to absorb out phenolic extracts from the nodule lobes which may hinder the proper growth of the endophytes (Thomas 2008). After incubation, the nodule lobes were washed twice with sterile distilled water to remove charcoal. Epidermal layers were removed and nodule lobes were chopped into small pieces. Each nodule piece was incubated with 10 mL of Qmod (Lalonde and Calvert 1979) medium in 30 mL screw cap test tube at 28 °C for around one month in dark without shaking.

After one-month incubation in dark, bacterial colonies formed minute cottony-white dots at the bottom of the tube. Each colony was collected and subcultured in separate tubes containing Qmod medium.

2.2 Culture growth conditions

After successful isolation of the bacteria in pure culture, the isolate was maintained in Qmod medium in 250 ml flask in dark with occasional shaking.

2.3 Field emission scanning electron microscopy

For field emission scanning electron microscopy, one-month-old *Prauserella*, cultures were used. The bacterial cells were harvested in a micro-centrifuge tube and centrifuged at 1000 g for 5 min. The cells were washed with distilled water and treated with 4 % (*w*/*v*) glutaraldehyde solution in phosphate

buffer (pH 7.2) for 4 h at 30 °C. The glutaraldehyde solution was decanted and the samples were washed with the same buffer for three times. The samples were treated with 1 % Osmium tetra oxide solution in phosphate buffer (pH 7.2) for 1 h and washed with the phosphate buffer (pH 7.2) for 3 h. The *Prauserella* samples were treated bypassing through with different graduated ethanol solutions from 50 % to absolute for 10 min each. The 95 % ethanol wash was performed twice. After dehydration, the samples were stored in absolute alcohol for microscopy (Bajwa 2004).

The samples were critical point dried for 2 h. Each sample was mounted on copper tape and platinum coated (by JFC 1600 platinum caster, JEOL, Japan) and viewed on JEOL JSM-6700F field emission scanning electron microscope at an accelerating voltage of 2KV.

2.4 Plant infectivity test

The seeds of healthy *A. nepalensis* were collected in the month of March from Darjeeling hills. The seeds were soaked overnight with double distilled water. The soaked seeds were surface sterilized with 30 % H_2O_2 for 10 min followed by washing several times with sterile double distilled water. The seeds were placed on a sterile moist filter paper on a Petri plate with 1X Hoagland solution (Hoagland and Arnon 1950) and kept in a seed germinator at 30 ± 1 C for germination. Fifteen days old seedlings were transferred to a magenta box containing sterile N_2 free Hoagland solution on sterile blotting paper supported by stainless steel supporter. The following three sets were prepared for each isolates: (1) seedlings inoculated with crushed nodule suspension (positive control); (2) seedlings inoculated with 100 μl of 30 day old culture; (3) un-inoculated seedlings (negative control). Twenty seedlings were used in each set. After an incubation period of 20–30 days, all the seedlings were carefully examined to detect any root hair deformation or nodule formation.

2.5 Genome sequencing and assembly

The draft genome of *Prauserella* strain Am3 was generated at the Hubbard Genome Center (University of New Hampshire, Durham, NH) using Illumina technology (Kuhn et al. 2004) techniques. A standard Illumina shotgun library was constructed and sequenced using the Illumina HiSeq2000 platform, which generated 29,139,048 reads (260 bp insert size) totaling 4370.9 Mbp. The Illumina sequence data were assembled using CLC Genomics Workbench (6.5.1) and AllPaths-LG (Version r41043) (Gnerre et al. 2011). The final draft assembly contained 61 contigs with an N_{50} of 370 kb. The total size of the genome is 5.3 Mbp, and the final assembly is based on 2057.3 Mb of Illumina draft data, provided an average 680 x coverage of the genome.

Genome annotation was performed within the Integrated Microbial Genomes (IMG) platform developed by the Joint Genome Institute, Walnut Creek, CA, USA (Markowitz et al. 2006). Genes were identified using Prodigal (Hyatt et al. 2010). The predicted CDSs were translated and used to search the National Center for Biotechnology Information (NCBI) non redundant database, UniProt, TIGRFam, Pfam, KEGG, COG, and InterPro databases. The tRNAScanSE tool (Lowe and Eddy 1997) was used to find tRNA genes, whereas ribosomal RNA genes were found by searches against models of the ribosomal RNA genes built from SILVA (Pruesse et al. 2007). The metabolic potential of the genome was analyzed by the use of the antiSMASH program (Blin et al. 2013; Medema et al. 2011) and IMG platform.

2.6 Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JTJI00000000. The version described in this paper is version JTJI01000000.

2.7 Bioinformatics study

2.7.1 Retrieval of genome sequences

The nucleotide sequences along with the translated protein sequences of 21 *Amycolatopsis* and 2 *Prauserella* genomes were retrieved from JGI-IMG database (http://img.jgi.doe.gov/) (Mavromatis et al. 2009). A list of the genomes and general properties are presented in supplementary Table 1. Major information regarding the strand specificity and functional Cluster of Orthologs (COG) category (Tatusov et al. 2000) for each gene were also obtained from the above mentioned database.

2.7.2 16s rRNA gene and MLSA phylogeny tree

The 16s rRNA genes for the 21 *Amycolatopsis* strains and two *Prauserella* strains were collected from IMG database. The partial 16s rRNA sequence for remaining *Prauserella* strains were collected from NCBI database. The 16s rRNA sequences were truncated to match the sequence length. This drastically reduces the unaligned regions too. The truncated 16s rRNA gene sequence alignment was done by ClustalW (Li 2003). Mega ver. 6 software (Tamura et al. 2007) was used to build the 16s rRNA gene based tree using neighbor-joining model and a bootstrap value of 1000. The Multi-Locus Sequence Analysis (MLSA) tree was generated with Random Axelerated Maximum Likelihood (RAxML) (Stamatakis 2006) software. For MLSA analysis, AtpI, GyrA, FtsZ, SecA, DnaK proteins were used. Although there are several

housekeeping proteins present in actinobacteria group, the five proteins were chosen because they represent single proteins universally found among all of the studied strains. The amino acid sequences of the five housekeeping genes were aligned by the use of ClustalW software (Li 2003) and alignment files were concatenated by Seaview software (Gouy et al. 2010). The concatenated file was used to make the tree in RAxML software with PROTGAMMAWAG (de Mendoza et al. 2010) substitution model and 1000 bootstrap value. *Pseudonocardia* was used as outgroup in these phylogenetic study.

2.8 ANI score and genome-to- genome distance calculation

Average nucleotide Identity (ANI) of several genomes was performed to reveal the genomic closeness. The ANI score calculates the similarity index between a given pair of genomes. If the score is >95 %, they are considered the same species (Kim et al. 2014). The ANI score was calculated via the ANI calculator tool developed by Kostas Lab (http://enve-omics.ce.gatech.edu/ani/). The Genome-to Genome distance calculator was also used to show the genomic distances among the four strains (2 *Prauserella* and 2 *Amycolatopsis*). The distance was calculated by GGDC 2.1 software (http://ggdc.dsmz.de/distcalc2.php). This tool calculates the genomic distance and transforms these values analogous to DNA-DNA Hybridization (DDH) values (Meier-Kolthoff et al. 2013). Generalized Linear Model (GLM) is used to estimate the confidence level of the DDH values. If the DDH value > = 70 %, the target and query genomes are consider to be same species. If the DDH value > = 79 %, they are considered to be the same sub-species (Meier-Kolthoff et al. 2013).

2.8.1 Amino acid and codon usage count

The codon and amino acid usage patterns were determined and compared among the studied genomes. A simple BioPerl module implemented in CMG Biotools (Vesth et al. 2013) was used to evaluate the codon and amino acid usage patterns. From that module, the third position codon bias (bT) was calculated by the following formula:

$$bT = \frac{No.\text{of each base on each position of each codon}}{\text{total number of codons}}$$

Two separate heat maps were generated on amino acid usage and codon usage by R package (Ihaka and Gentleman 1996). Rose plots were also generated to have a deeper look on the amino acid and codon usage preference of each genome under investigation (Vesth et al. 2013).

2.8.2 Identification of putative horizontally transferred (pHGT), pseudogenes and insertion sequence

Comparative analysis of the *Prauserella* genomes was performed using the IMG system (Markowitz et al. 2006). Putative horizontally transferred genes (pHGT) were defined as genes that have the best hits to genes that are not in the phylogenetic group of the query sequence. To determine a pHGT, not only was the best hit (highest bit score) used, but all of the hits that have bit score equal or greater than 95 % of the best hit were taken under consideration. Pseudogenes were identified on JGI-IMG platform via “Pseudo genes” option under the tab “Genes total number” (Markowitz et al. 2012). IMG database actually calculate the Pseudogene number via GenePRIMP (Pati et al. 2010). Insert sequences (IS) were identified via IS Finder server (Siguier et al. 2006).

2.8.3 Dot-plot generation

Dot Plots were generated via Mummer (Armengol et al. 2003) on the IMG site. The DNA sequences for two genomes were chosen and the nucmer (NUCleotide MUMmer) pipeline was used to generate the plots. The whole genome alignment of two *Prauserella* and *A. halophila* genomes was done by Mauve software. The circular genome view of *Prauserella* genomes were generated by GC view (Stothard and Wishart 2005) and have been given in supplementary files (ESM 1,2,3).

2.8.4 Creation of BLAST matrix and Pan Core plot

The BLAST matrix is a visual representation of a pairwise comparison of targeted proteomes using the BLAST algorithm (Vesth et al. 2013). The whole process of creating a blast matrix was performed on the CMG-Biotool platform using “matrix_createconfig” and “matrix” programs.

The pan and core genome analyses were also done by pancoreplot_createconfig (Vesth et al. 2013) program implemented in the CMG platform. The concatenated core gene family tree with Manhattan distance was built by pancoreplot tree program.

3 Results and discussion

3.1 Isolation of an actinobacterium from *Alnus* root nodules

The organism was isolated from root nodules of the *Alnus nepalensis* and it was identified as *Prauserella* by 16s rRNA gene analysis. An electron micrograph (Fig. 1) shows that the bacterium has fragmented mycelium. The mean diameter of the hyphae was found to be ±0.3 μm.

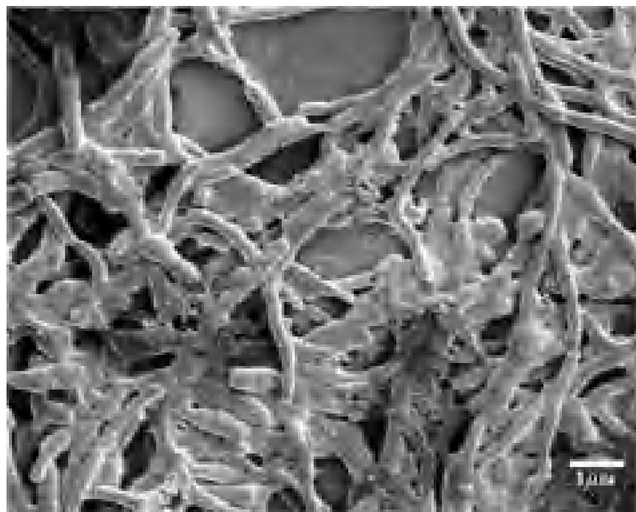

Fig 1 Scanning electron micrograph of *Prauserella* sp. Am3. The size bar represent 1 μm

3.2 Plant infectivity

We attempted to re-infect the host plant *A. nepalensis*, but failed to produce either root hair deformation or nodules on these root. The positive controls produced nodulated plants suggest that our test was okay. However, this result is not surprising. Most of the non-*Frankia* actinobacteria isolated from actinorhizal nodules are unable to re-infect their host plant.

3.3 Phylogenetic analysis of the isolates

A multi-tiered approach was used for phylogenetic analysis. First, a neighbor-joining (NJ) 16s rRNA gene phylogenetic tree grouped *P. rugosa* DSM 43194 and *Prauserella* sp. Am3 together with *A. halophila* YIM 93233 and *A. orientalis* DSM 43388. All of the partially sequenced 16s rRNA from different *Prauserella* strains formed one clade. However, all of the other *Amycolatopsis* strains were placed at a considerable distance from *P. rugosa* and *Prauserella* sp. Am3 (Fig. 2). Next, we determined the ANI scores among *A. halophila* YIM 93233, *A. orientalis* DSM 43388, *P. rugosa* and *Prauserella* sp. Am3 (Supplementary Table 2). *A. halophila* was closer to both *Prauserella* genomes than to *A. orientalis* DSM 43388. An in silico DNA-DNA hybridization via the Genome-to- Genome Distance calculator indicates that *Prauserella* sp. Am3 and *P. rugosa* may be the same species [DDH = 96.22 %].

Because of this close clustering pattern found with *A. halophila* YIM 93233, *A. orientalis* DSM 43388, *P. rugosa* and *Prauserella* sp. Am3, a phylogenomic study was performed by the use of a MLSA tree as described by Sen et al. (2014). The MLSA amino-acid-sequence-based tree also placed *A. halophila* YIM 93233 with both *P. rugosa* and *Prauserella* sp. Am3 in one clade with a good bootstrap value (Fig. 3). However, *A. orientalis* DSM 43388 was distantly placed with the other *Amycolatopsis* strains. This phylogenomic evidence supports the idea that *A. halophila* is somewhat closer to *Prauserella* than other *Amycolatopsis* strains. A comparative genomic summary of these three strains has been given in Table 1.

3.4 Amino acid and codon usage analysis

The codon and amino acid usage patterns of the genomes were determined and are shown in heatmaps (ESM4). All of the selected genomes showed a distinct GC bias. The most widely used codons were: GCG, GCC (alanine); GTC, GTG (valine); CGG, CGC (Arginine); CTC, CTG (Leucine); GAG, GGC (Glycine). Bioinformatics analysis of newly sequenced *Prauserella* sp. Am3 indicates the use of aliphatic amino acids with low energy cost and a preference of either G or C at the 3rd position of the codon. Thus, GC percentage and GC3 percentage are obviously governing the codon usage pattern in *Prauserella* sp. Am3.

3.5 Major shifts and realignments among *Prauserella* genomes

To understand the presence of major shifts and realignments, the *Prauserella* genomes were analyzed by the use of a DNA-DNA dot plot (Fig. 4). Both *Prauserella* genomes were very similar and showed no signs of translocation. These results along with results from the ANI and GGDC scores described above indicating that *Prauserella* sp. Am3 strain may be a member of *P. rugosa* species.

3.6 Genome plasticity

The two *Prauserella* and *A. halophila* genomes were analyzed for the presence of pHGT, IS elements and pseudogene (ESM5). pHGTs were approximately 0.68 % and 1.03 % of gene content of the *P. rugosa* and *Prauserella* sp. Am3 genomes, respectively. However, no pHGTs were identified in the *A. halophila* genome which might be due to nature of minimum draft sequence data. The percentage of IS elements present in *P. rugosa*, *Prauserella* sp. Am3 and *A. halophila* genomes were 5.26, 5.53 and 5.36 %, respectively. This result suggests that the selected genomes are not very stable and show high degree of plasticity. Pseudogenes were not identified in either of the two *Prauserella* genomes. Insertion sequences play an important role in evolution as they can promote the genome plasticity and gene inactivation (Touchon and Rocha 2007). pHGT generally is a positive indicator of IS element amount (Touchon and Rocha 2007).

Fig 2 16s rRNA gene based Neighbour joining tree of *Prauserella* and *Amycolatopsis* genomes with1000 bootstrap values represented as percentage. Two strains of *Prauserella* have clustered with *A. halophila.* The (T) designate type strains and * designate genes taken from NCBI. Other genes are taken from IMG database. Corresponding Genbank accession numbers have been given for the sequences obtained from NCBI and for others Locus Tag has been given within parenthesis

3.7 Strand variation property

The strand-biased properties of the two *Prauserella* and *A. halophila* genomes were analyzed and visualized by a bar diagram (ESM 6). Stringent strand asymmetric bias was not observed (regardless of their expression pattern). The differences between the two *Prauserella* strains regarding the strand specificity nature may result from inversion mutations which have the potentiality to convert the leading strand genes to lagging strand genes as proposed by Chen and Zhang

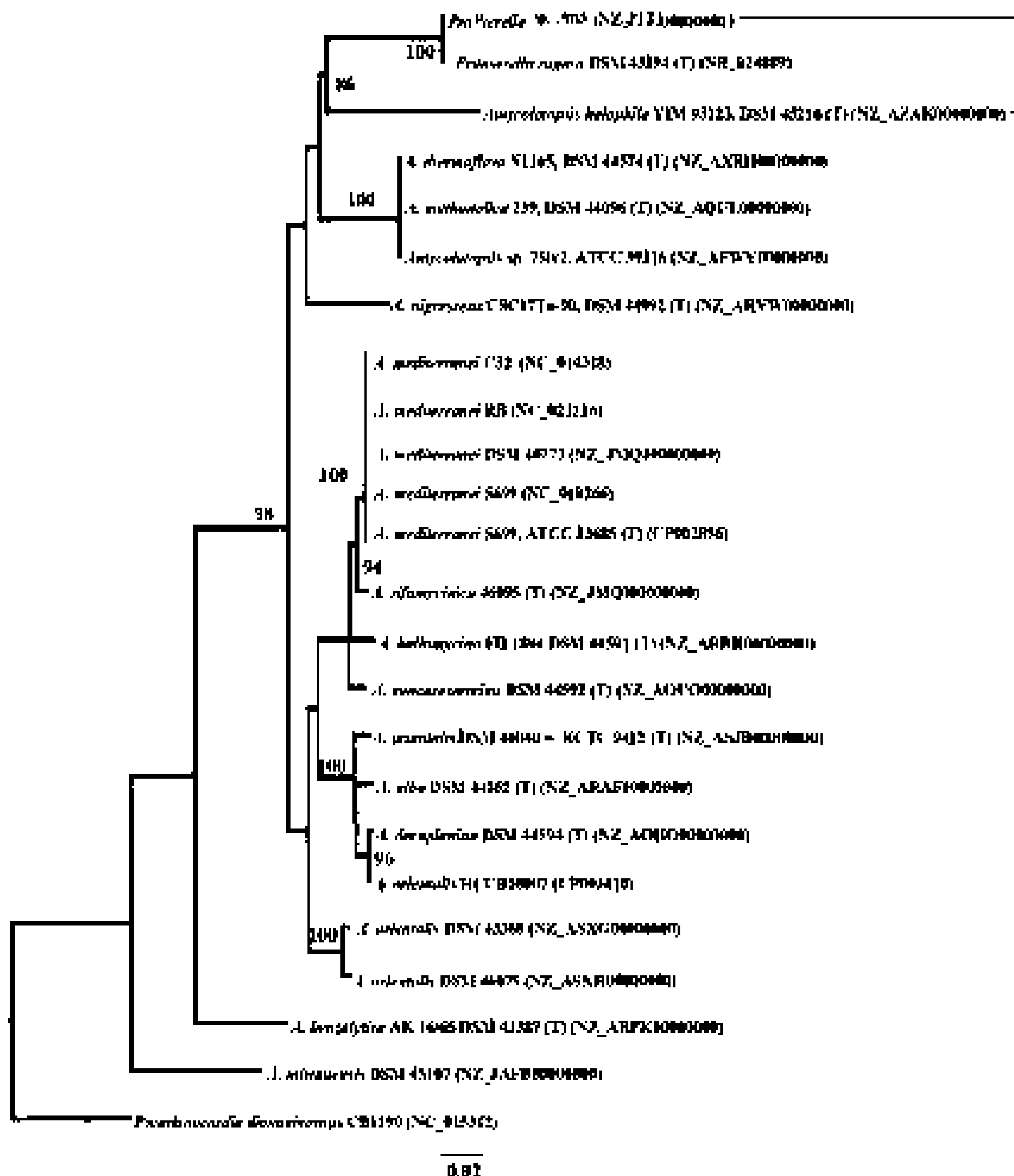

Fig 3 MLSA protein based tree of *Prauserella* and *Amycolatopsis* genomes built by RAxML using PROTGAMMAWAG substitution method and 1000 bootstrap value. Bootstarp values were represented in percentage. Two strains of *Prauserella* have clustered with *A. halophila* indicated by *ash box*. The (T) designate type strains and genbank accession numbers are given inside the parenthesis

(2013). However, it still needs a detailed study. Thus, absence of replication associated mutational pressure is confirmed on these genomes as there was no strand asymmetric nature persisted on those three considered genomes (Das et al. 2006). However, when we considered the potentially highly expressed genes, a considerable number of highly expressed genes were found to be located on the lagging strand in all three strains. These results indicate that transcriptional-associated mutational pressure was not apparent. This type of result was also found in *Bifidobacteria* (Roy et al. 2015), another group of actinobacteria living in the gut of animal and human host. Chen and Zhang (2013) proposed that, some microbial genomes have highly-expressed genes on their lagging strand for attaining proper mutation-selection balance and our data can be supported by this theory.

Table 1 Summary of genome properties

Characterization	*Prauserella rugosa* DSM 43194	*Prauserella* sp. Am3	*Amycolatopsis halophila* DSM 45216
Accession Number	NR_024889	NZ_JTJI00000000	NZ_AZAK00000000
Genome Size(bp)	5,220,823	5,321,061	5,550,897
Scaffolds	2	46	1
GC content (%)	70	70	68
Gene content	4985	4969	5187
No. of genes for:			
rRNA	9	8	6
tRNA	48	47	49
CDS	4923	4907	5187
Biosynthetic cluster count	62	53	54
Biosynthetic cluster gene count	689	593	817
NCBI Taxon ID	43,354	1,515,610	592,678
No. of CRISPR	2	1	-

3.8 Pan- and core- genome plot

The pan and core-genomes for *Amycolatopsis* and *Prauserella* were determined as described in the Methods. As expected, with the addition of the further genomes, the pan-genome (blue circles) increased due to the accumulation of accessory genes whereas the conserved core genome (red circles) decreased. The core-gene pool contained genes which are essential for transcription, translation, replication and metabolism. A total of 1589 genes were found to be in the core genome for the 21 *Amycolatopsis* and 2 *Prauserella* strains and the pan genome contained 35,441 genes (ESM 7). Manhattan-distance-based concatenated phylogenetic tree based on the core genome (1589 genes) was build (ESM 8). The *Prauserella* genomes clustered with *A. halophila* and were separated from the rest of *Amycolatopsis* genomes. To get a clear picture of these genomes, we focused on pan and core-genomes of the two *Prauserella* and *A. halophila* strains. The distribution of commonly shared genes is shown in Venn diagrams (Fig. 5). The two *Prauserella* genomes shared a total of 4224 genes (Fig. 5a core gene indicated by black box). When the *A. halophila* genome was included in the analysis, this core-genome was reduced to 2124 genes (Fig. 5b, core gene indicated by black box).

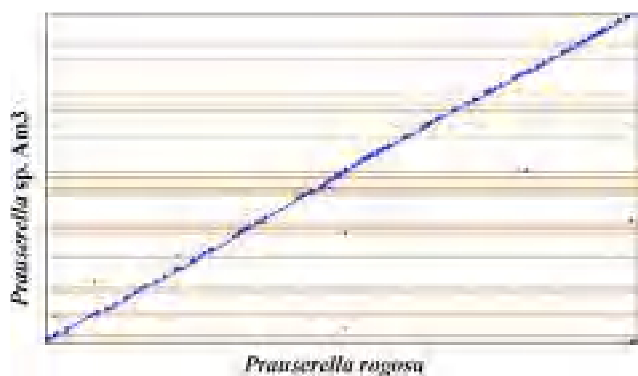

Fig 4 DNA-DNA dot plot of *Prauserella rugosa*, *Prauserella* sp. Am3 showing a high similarity between these two genomes

3.9 Blast matrix

The blast matrix of these genomes was generated by CMG biotools. The percentage of similarity among different genomes was represented with green color and the homology within a strain was visualized by red color (ESM 9). Two

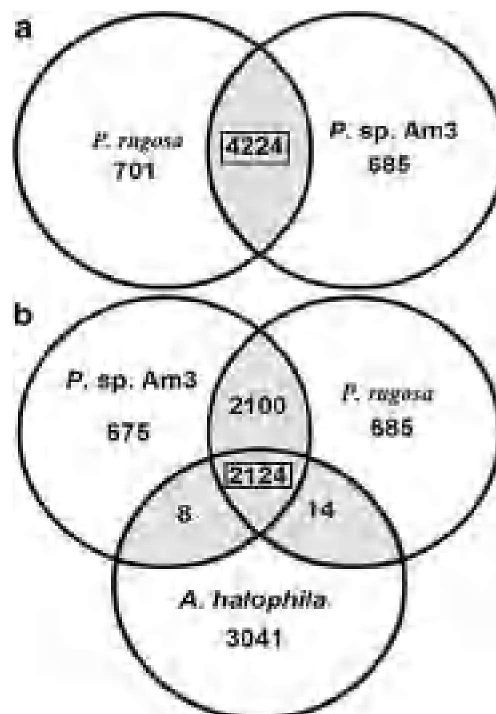

Fig 5 Venn diagrams of core and unique genes (**a**) between two *Prauserella* genomes and (**b**) among two *Prauserella* genomes and *A. halophila*

Prauserella genomes shared 85.7 % homology with each other. Though, blast matrix did not show a very high similarity between the *Prauserella* genomes and *A. halophila*, the percentage of similarity were more than the similarities obtained between *A. halophila* and other *Amycolatopsis* strains.

3.10 Proteins of Major Functional categories among investigated organisms

The predicted proteins were classified according to their Cluster of Orthologs (COG) category (Tatusov et al. 2000) and the results are presented in supplementary figure ESM 10 showing their distribution among the genomes. Most of the proteins were classified into the following COG categories: E (Amino acid transport and metabolism), G (Carbohydrate transport and metabolism), C (Energy production and conversion), and K (Transcription), which are mainly associated with the essential functions for an organism to survive in nature.

4 Conclusion

In this study, we have sequenced a new strain of *Prauserella* and have compared its genome with the type strain of the genus *P. rugosa* and other selected *Amycolatopsis* genomes, which are were reported as the closest genus of *Prauserella*. From both the 16s rRNA gene sequence based phylogenetic and MLSA amino acid sequence based phylogenomic analyses, *P. rugosa* and *Prauserella* sp. Am3 were found to be closer to *A. halophila* rather than any other *Amycolatopsis* strains. There were 4224 shared genes (core genome) between the two *Prauserella* genomes, which reduced to 2124 genes when the *A. halophila* genome was included. Analysis of the DNA-DNA dot plot revealed a high degree of similarity between the two *Prauserella* genomes.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Funding This work is also partially supported by Department of Biotechnology, Govt. of West Bengal, India through grant no. 206/Bt(Estd.)/RD-22/2014. LST is supported in part by a USDA National Institute of Food and Agriculture Hatch 022821.

Conflict of interest The authors declare that they have no conflict of interest.

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GenBank

Camellia sp. RL-2020 isolate Thurbo 9 maturase K (matK) gene, partial cds; chloroplast

GenBank: MN480322.1

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  TITLE     Direct Submission
  JOURNAL   Submitted (17-SEP-2019) Molecular Cytogenetics Laboratory,
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Nucleotide

GenBank

Camellia sp. RL-2020 isolate Thurbo 3 maturase K (matK) gene, partial cds; chloroplast

GenBank: MN480321.1

FASTA Graphics

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  AUTHORS   Labar,R., Bhattacharya,M., Kar,P., Biswas,P. and Sen,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-SEP-2019) Molecular Cytogenetics Laboratory,
            Department of Botany, University of North Bengal, Rajarammohunpur,
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Nucleotide

GenBank

Camellia sinensis clone Takdah 383 maturase K (matK) gene, partial cds; chloroplast

GenBank: MK424872.1

FASTA Graphics

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  JOURNAL   Submitted (23-JAN-2019) Molecular Cytogenetics Laboratory,
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Nucleotide

GenBank

Camellia sinensis clone Bannockburn 157 maturase K (matK) gene, partial cds; chloroplast

GenBank: MK424871.1

FASTA Graphics

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  JOURNAL   Submitted (23-JAN-2019) Molecular Cytogenetics Laboratory,
            Department of Botany, University of North Bengal, Rajarammohunpur,
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GenBank

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GenBank: MK424870.1

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REFERENCE   1  (bases 1 to 755)
  AUTHORS   Bhattacharya,M., Labar,R., Kar,P., Biswas,P. and Sen,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JAN-2019) Molecular Cytogenetics Laboratory,
            Department of Botany, University of North Bengal, Rajarammohunpur,
            Siliguri, West Bengal 734013, India
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Nucleotide

GenBank

Camellia sinensis clone Tocklai Variety 19 maturase K (matK) gene, partial cds; chloroplast

GenBank: MK424868.1

FASTA Graphics

Go to:

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LOCUS       MK424868                 750 bp    DNA     linear   PLN 21-AUG-2019
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VERSION     MK424868.1
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            Pentapetalae; asterids; Ericales; Theaceae; Camellia.
REFERENCE   1  (bases 1 to 750)
  AUTHORS   Bhattacharya,M., Labar,R., Kar,P., Biswas,P. and Sen,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JAN-2019) Molecular Cytogenetics Laboratory,
            Department of Botany, University of North Bengal, Rajarammohunpur,
            Siliguri, West Bengal 734013, India
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Nucleotide

GenBank

Camellia sinensis clone Phoobsering 1258 maturase K (matK) gene, partial cds; chloroplast

GenBank: MK393404.1

FASTA Graphics

Go to:

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VERSION     MK393404.1
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REFERENCE   1  (bases 1 to 751)
  AUTHORS   Kar,P., Labar,R., Bhattacharya,M., Biswas,P. and Sen,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-JAN-2019) Molecular Cytogenetics Laboratory,
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26th West Bengal State Science & Technology Congress, 2019

Organised by

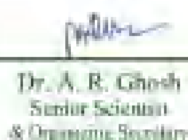
Department of Science & Technology and Biotechnology, Government of West Bengal

The Paper titled *Phytochemical and antimicrobial screening of selected Darjeeling based On varying Polarities of extracting solvents*

authored by *Reha Labar, Mulay Bhattacharya, Arnab Sen*

presented by Prof./Dr./Smt./Sri *Reha Labar*

is awarded as 'Outstanding Paper' in the 26th West Bengal State Science and Technology Congress, 2019 held on 28th February / 1st March, 2019, at Science City, Kolkata.



Dr. A. R. Ghosh
Senior Scientist
& Organising Secretary

Dr. S. Kishore, IAS
Principal Secretary

Bratya Basu
Minister-In-Charge

UNIVERSITY OF NORTH BENGAL

Accredited by NAAC with Grade A

International Seminar on "Frontiers in Tea Research-2020"

March 6, 2020

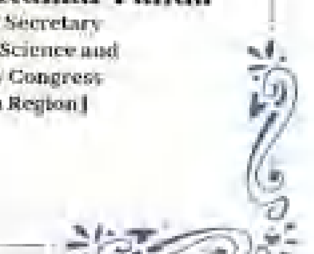
Organized by

DEPARTMENT OF TEA SCIENCE

This is to certify that Prof./Dr./Mr./Mrs/Miss. REHA LABAR of Dept. of Botany, NBU has delivered an invited Lecture/Oral presentation/Poster presentation/participated in the one day International Seminar on "Frontiers in Tea Research-2020" held at the University of North Bengal on 6th March, 2020.

He/She has presented a paper titled "DNA barcode profilling..... consensus region"

Prof. Pranab Ghosh **Chairman** **Organizing Committee**	Dr. Chandra Ghosh Head, Department of Tea Science **Convener, Organizing Committee**	Dr. M. Bhattacharya & Dr. Sonali Ray **Conveners** **Organizing Committee**



3rd Regional Science & Technology Congress - 2018

West Bengal, (Northern Region)

Organised jointly by

Jalpaiguri Government Engineering College

and

Department of Science and Technology and Biotechnology, Government of West Bengal

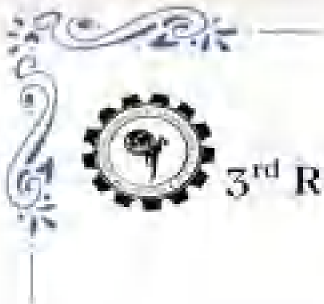
Certificate of Outstanding Paper

This is to certify that the Paper titled *Phytochemical and antimicrobial Screening of selected Darjeeling tea clones based on varying polarities of extracting Solvents.* Authored by *Sneha Labar* has been adjudged as Outstanding Paper in the session of *Botany* presented in the 3rd Regional Science & Technology Congress - 2018, West Bengal (Northern Region) held on 12th & 13th December, 2018 at Jalpaiguri Government Engineering College, Jalpaiguri, West Bengal, India.

Dr. Bimalesh Samanta
Senior Scientist
Dept. of Science and Technology and Biotechnology, Government of West Bengal and Nodal Officer & Joint Organising Secretary
3rd Regional Science and Technology Congress
[Northern Region]

Dr. Amitava Ray
Principal
Jalpaiguri Govt. Engg. College

Prof. Goutam Kumar Panda
Organising Secretary
3rd Regional Science and Technology Congress
[Northern Region]



NATIONAL SEMINAR ON NEW HORIZON IN BOTANICAL RESEARCH

FEBRUARY 20-21, 2020

ORGANISED BY

DEPARTMENT OF BOTANY
UNIVERSITY OF NORTH BENGAL
Accredited by NAAC with Grade A

CERTIFICATE

This is to certify that Prof. / Dr. / Mr. / Ms. Reha Labar

of Botany *participated / chaired a session/*

presented a paper in oral/~~poster session~~/entitled Effect of solvent with varying polarities on phytochemical extraction .....in-silico approaches *in the above seminar.*

Prof. Aniruddha Saha
Organizing Secretary
BOTSEM- 2020

Prof. Subhas Ch. Roy
Chairman
BOTSEM-2020