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RESEARCH ARTICLE

COMPARATIVE GENOTOXIC EFFECT OF GREEN AND CURED LEAVES OF CHEWING TOBACCO ON *ALLIUM CEPA* CHROMOSOMES

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SUMMARY Genotoxic potential of chewing tobacco (*Nicotiana tabacum*) leaf of both fresh and cured form has been assessed by exposing the anthers of *Allium cepa* in their respective prepared extracts for different duration. The study revealed the genotoxicity of both green and cured leaves of chewing tobacco i.e. khaini, most commonly used smokeless tobacco in the region of Jharkhand.

Keywords: *Nicotiana tabacum*, smokeless tobacco, genotoxicity, *Allium cepa.*

INTRODUCTION

Nicotiana tabacum, is the world's commercially cultivated non-food cash crop. It is a perennial herbaceous plant belonging to family Solanaceae. Plants are grown mainly for its leaves, used commercially in the production of cigarettes, chewing tobacco and nicotine replacement products. *N. tabacum* also known as desi types in India have tall plants with broad leaves and usually have pink flowers. Tobacco is known for the foremost cause of most preventable deaths. According to global adult tobacco survey reports (GATS 2017), India is the world's second largest producer of tobacco and also second largest consumer of processed tobacco. Beedi, cigarette and chewing tobacco are the major forms that are consumed in India while smoking constitutes a small part of tobacco related problem but large part of consumption is the oral use of smokeless tobacco products. According to WHO, currently, there are 267 million tobacco users in India and the situation is more alarming than any other country with large consequential burden of tobacco related disease and death.

Smokeless tobacco (ST) refers to the use of tobacco without burning or heating at the time of use. It can be used orally or nasally. The oral use of ST is widely prevalent in India and according to global adult tobacco survey reports 21.4% (men 29.6% and women 12.8%) of adults are using ST. It can be used in a variety of ways like gutka chewing, sucking and applying tobacco preparations to the teeth and gums. Aggressive marketing, longer shelf-life and its cost effectiveness lead to a dramatic increase in the habit of chewing tobacco, even among women and children.

Khaini a type of chewing tobacco is most commonly used ST in Bihar and Jharkhand. It is a mixture of tobacco and slaked lime. Though nicotine is not only toxic chemical present in the

ST, it is mainly responsible for addiction. Khaini and zarda contain high level of carcinogenic compounds like N-Nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone (NNK), N9-nitrosoanabasine (NAB) and N-nitrosoanatabine (NAT) as compared to the green tobacco (Bhisey 2012). The concentration of these chemicals may get increased during the curing process of tobacco leaves (Bhide et al. 1987). Most of the research on global basis is only concerned with the use of tobacco as cigarette smoking but as far as developing country like India is concerned, it is facing and in future also going to face a lot more complexity regarding the consumption of various forms of tobacco products especially khaini in backward states. Besides harmful usage of tobacco leaves in smoking and chewing, it was known for its medicinal values. In India and China, raw green tobacco leaves were used for treating painful piles and rheumatic swelling (Agyare et al. 2013). Since ancient times these plants are used by Peruvian Amazonian community for treating mental illness, parasitic illness and respiratory problems, specially *N. rustica* is described as master plant in this region. The green tobacco preparation was administered in either liquid or solid form via oral, tropical intranasal and rectal note. Although there are many reports about the neuropsychiatric disorders caused by green tobacco, still it is used in its native place because it was a notion that green tobacco is having spiritual energetic property (Berlowitz et al. 2020). On a small scale, these green tobacco extracts are also used by local farmers as green pesticides and used to kill pests like aphids, jassids and whiteflies. The high nicotine content is having insecticidal properties (Gudeta et al. 2021).

Inspite of useful properties of green tobacco leaves, its cytotoxic and genotoxic effect cannot be ignored. It is as toxic as cured form i.e. smokeless tobacco (khaini) and cigarette. Few research papers focus on occupational hazard of green tobacco leaves responsible for green tobacco sickness (GTS) prevalent in Asian and South American tobacco harvesters. The sickness occurs due to the intradermal absorption of nicotine from the wet surface of tobacco plants. GTS symptoms include nausea, vomiting, dizziness, delirium, increased perspiration, abdominal pain, diarrhoea, increased salivation, weakness, breathlessness and occasional lowering of blood pressure (Shailee & Fotedar 2017). Vomiting can lead to dehydration and adds to the risk of heat illness. These symptoms are so common that GTS remains ignored and so not well documented. Tobacco is grown in more than 100 countries and tobacco processing is all done manually and there is extended exposure of GTS which may lead to diseases like cardiovascular disease or may have mutagenic effect which manifested as cancer (Mc Bride et al. 1998). These symptoms are so common and similar to narcotic drugs but GTS remains ignored and not well documented. The present study is intended to add an additional evidence to the genotoxic potential of tobacco leaf extracts and it will also open a new sphere to explore the genotoxic potential of fresh green tobacco leaf extracts.

MATERIAL AND METHODS

The young and healthy flower buds of *Allium cepa* were used as a test material and chewing tobacco extracts of both green leaves and cured leaves i.e. khaini, popularly consumed form of chewing tobacco in Jharkhand and Bihar as test

substance. Seeds of *N. tabaccum* were procured from Dr. Rajendra Prasad Agricultural University, Pusa, Samastipur, Bihar for raising plants. Cured leaves were also procured from local market of Pusa, Samastipur. Extracts of both green and cured leaves were prepared in distilled water. 100 g of cured leaves were crushed and soaked in 1000 ml of distilled water and filtered through Whatman filter paper while 100 g of green leaves were directly ground and mixed with 1000 ml of distilled water and strained.

Healthy flower buds of *A. cepa* were exposed to the green tobacco leaf extract for half an hour, 1 h, one and a half h and 2 h. Similarly, buds were also exposed to cured leaf extract for the same duration. After exposure, buds were fixed in Carnoy's fixative (absolute alcohol and acetic acid in the ratio 3:1) for 24 h supplemented with a little pinch of ferric chloride as mordant, and then transferred in 70% alcohol for preservation. Excised anthers were stained in acetocarmine and slides were prepared by squash technique and observed under Magnus s/n: C197050239 microscope. Photographs were taken at 40 × and 100 ×. Total abnormality percentage was calculated by using the following formula :

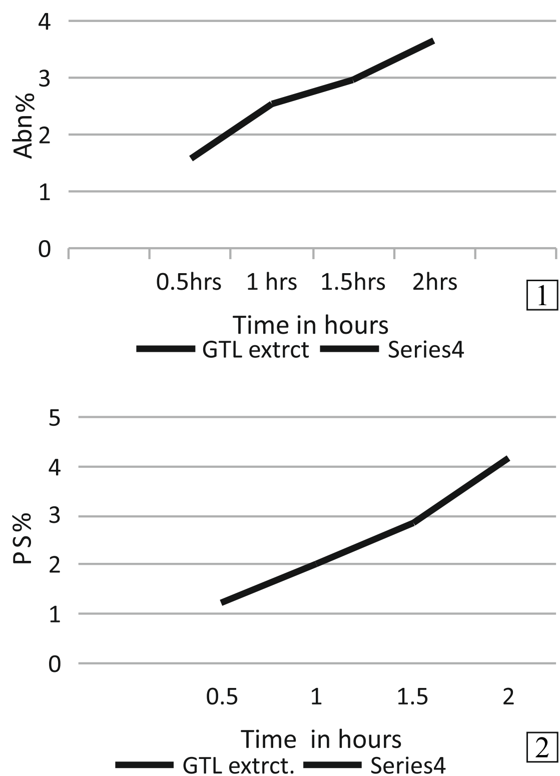
$$\text{Total abnormality percentage (Abn\%)} = \frac{\text{Total number of abnormal cells}}{\text{Total cell count}} \times 100$$

Percentage of pollen sterility was calculated by using the following formula:

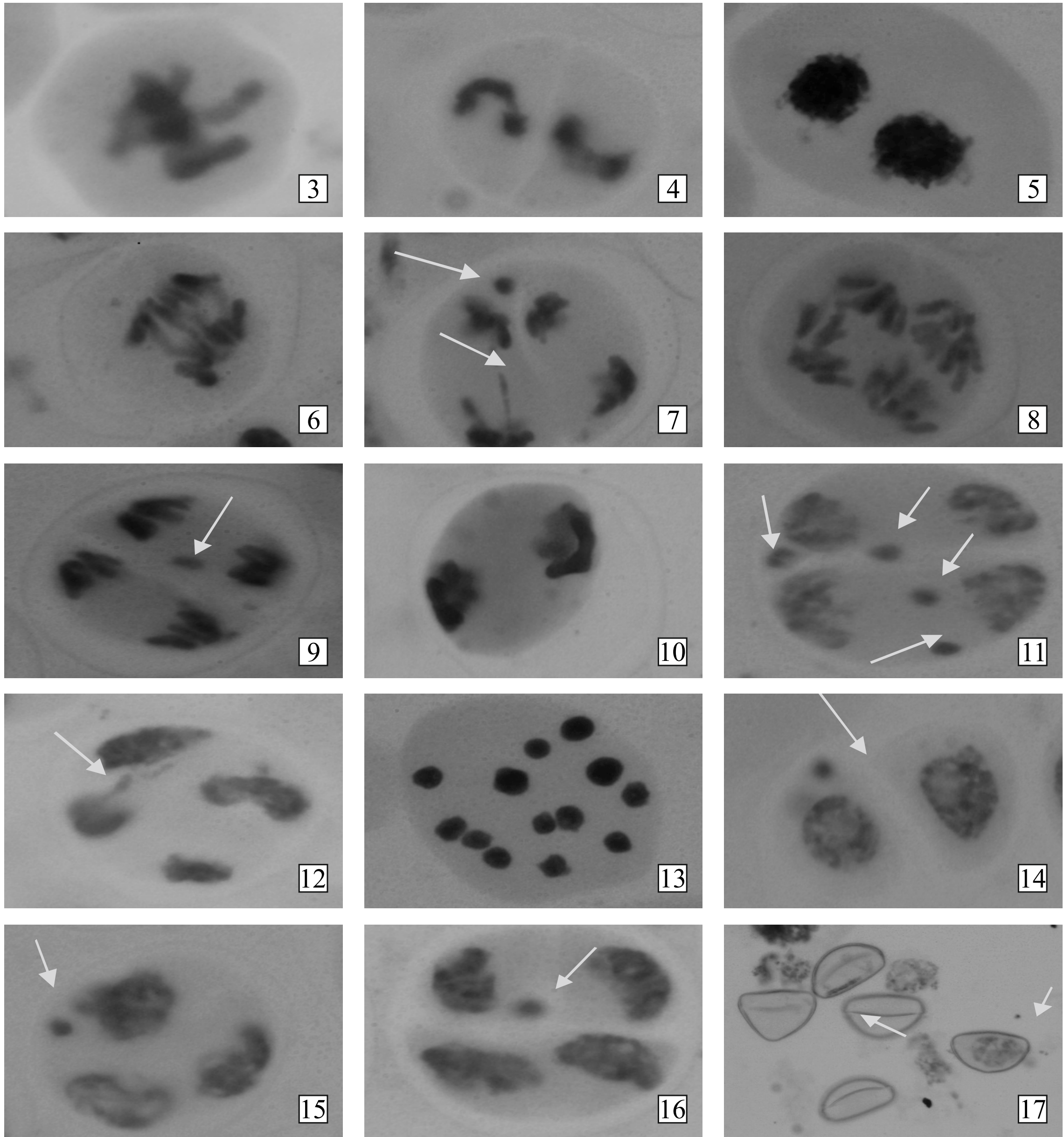
$$\text{Pollen sterility percentage (PS\%)} = \frac{\text{Total number of abnormal cells}}{\text{Total number of pollens examined}} \times 100$$

OBSERVATIONS

Microscopic examination of exposed young and mature flower buds of *A. cepa* showed different types of abnormalities as compared to those under control. In both green and cured samples, the increase of meiotic abnormalities was dependent on the increase in exposure time period as mentioned in Tables 1 and 2. The entire experiment was conducted in five replicates. It was observed that both green and cured forms induce genotoxicity (Figs 1, 2). The most common abnormality that was consistent in both forms of treatment is stickiness. It was observed in every stage of cell division (Figs 3–5). In addition, bridges and laggards at



Figs 1 & 2: *A. cepa* 1. Percentage of abnormality induced by green tobacco leaf extracts (GTL) and cured tobacco leaf extracts (CTL) 2. Percentage of pollen sterility (PS%) induced by GTL & CTL



Figs 3–17: *A. cepa* Meiotic abnormalities. 3. Cell at metaphase I showing stickiness of chromosomes. 4. cell at metaphase II showing stickiness of chromosomes. 5. Cell at anaphase I showing stickiness of chromosomes. 6. Cell at anaphase I showing multiple bridges. 7. Cell at anaphase II showing a bridge and micronuclei (arrows). 8. Cell at anaphase II showing multiple bridge. 9. Cell at anaphase II showing a laggard (arrow). 10. Cell showing stickiness and disturbed polarity at telophase I. 11. Cell at telophase II with multiple micronuclei (arrows). 12. Cell showing telophase II with a laggard (arrow). 13. Cell showing karyorrhexis. 14. Dyad with micronucleus (arrow). 15. Triad with micronucleus (arrow). 16. Tetrad with micronucleus (arrow). 17. Fertile and sterile pollen grains.

TABLE 1: Different types of M I and M II abnormalities due to green tobacco leaf extract.

Time	TCC	TAC	Abn% ± SD	Metaphase I/II	Anaphase I/II					Telophase I/II					Meiotic products		
				ST	ST.	BD	LG	DP	MN	ST	BD	LG	DP	MN	Dyad	Triad	Tetrad
Control	1250	00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	1250	20	1.6 ± 0.56	4	3	1	1	-	2	2	2	1	-	2	-	-	1
1	1250	32	2.56 ± 0.86	6	2	2	1	1	2	2	3	2	3	4	1	1	2
1.5	1250	37	2.96 ± 1.30	7	3	2	3	2	4	2	2	3	2	3	1	2	1
2	1250	46	3.68 ± 1.84	9	4	4	2	4	3	3	4	2	1	4	2	1	3

Abn% – Abnormality percentage, BD – Bridge, DP – Disturbed polarity, LG – Laggard, MN – Micronuclei, SD – Standard deviation, ST – Stickiness, TAC – Total abnormal cell, TCC – Total cell count.

TABLE 2: Different types of M I and M II abnormalities due to cured tobacco leaf extract.

Time	TCC	ACC	Abn% ± SD	Meta-phase I/II	Anaphase I/II				Telophase I/II						Meiotic products		
				ST	ST	BD	LG	DP	MN	ST	BD	LG	DP	MN	Dyad	Triad	Tetrad
Control	1250	00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	1250	45	3.6 ± 0.94	4	3	5	4	3	5	2	5	4	-	5	2	-	3
1	1250	59	4.7 ± 0.92	5	4	6	5	5	6	4	4	5	6	4	-	2	3
1.5	1250	66	5.2 ± 1.94	5	3	7	6	4	6	2	5	5	7	3	3	2	8
2	1250	85	6.8 ± 1.99	10	5	8	7	5	7	6	7	6	5	5	4	5	5

Abn% – Abnormal percentage, BD – Bridge, DP – Disturbed polarity, LG – Laggard, MN – Micronuclei, SD – Standard deviation, ST – Stickiness, TAC – Total abnormal cell, TCC – Total cell count.

TABLE 3: Percentage of pollen sterility due to green tobacco leaf extract.

Time	TNPE	TNSP	SP% ± SD
0.5	400	1	1.24 ± 0.79
1	500	1	2.00 ± 0.63
1.5	600	2	2.83 ± 0.833
2	600	0	4.16 ± 2.23

SD – Standard deviation, SP% - Percentage of pollen sterility, TNPE – Total number of pollen grains examined, TNSP – Total number of sterile pollen grains.

TABLE 4: Percentage of pollen sterility due to cured tobacco leaf extract.

Time	TNPE	TNSP	SP% ± SD
0.5	400	7	1.75 ± 1 .27
1	500	15	3.00 ± 1.414
1.5	600	35	5.83 ± 1.66
2	600	53	8.80 ± 1.632

SD – Standard deviation, SP% - Percentage of sterile pollen grains, TNPE – Total number of pollen grains examined, TNSP – Total number of sterile pollen grains.

both anaphase I and II, and at telophase II (Figs 6–9) disoriented telophase I (Fig.10) were observed. Exposure of anthers for a longer duration results in drastic change in cell structure, clearly showing its cytotoxic effect. A high frequency of aberrant microspore including monads, dyads, triads and tetrads with micronuclei and pollen sterility were frequently observed (Figs 14–17). Pollen sterility was reported in extract exposed anthers (Tables 3, 4).

DISCUSSION

Tobacco leaves are known to contain more than 4200 different chemicals ranging from heavy metals, polycyclic aromatic hydrocarbons to mutagenic chemicals (Rodgman & Perfetti 2009). Smokeless tobacco i.e. chewing tobacco (khaini) has become a substitute for cigarette smoking in Bihar and Jharkhand and it has been gaining interest and attention in the public health community and the tobacco industry. Smokeless tobacco is often promoted as safer than cigarette but reports suggest that it is as deleterious as smoking tobacco and chewing tobacco is leading to pancreatic, oral and oesophageal cancer (Boffetta et al. 2008). Cytogenetic tests are important in determining chromosomal abnormalities after exposure to the mutagen. It makes the chromosomes significant in the study of plant cell biology and other fields such as environment monitoring and genotoxicity studies (Kwansniewska & Bara 2022). Meiosis is a foundation of sexual reproduction in any organism. Plant models are useful in studying meiotic progression as they are known to have relaxed cell cycle as compared to animals and yeast, which accept different types of modifi-cations to their meiotic cycle (Wijnker & Schnittger 2013). In the present study, meiotic abnormalities such as bridges, stickiness, laggards, micronuclei, fragmentation, synchronization disorder, are induced by both cured and green tobacco leaf extracts. While stickiness was found to be most dominant anomaly at metaphase, chromosomal stickiness may occur due to interchromosomal linkage coupled with excessive nucleoprotein formation or alteration in their pattern of organization or due to depolymerization of nucleic acid. These deviations from normal process may be the consequences of extract treatment (Srivastava & Kapoor 2008). Chromosomal stickiness was first reported in maize and reports suggest that it was due to mutation caused by a recessive gene called sticky (*st*) (Beadle 1932). In acute stickiness there is lack of centromere and spindle interaction and chromosome separation which may lead to the formation of single or multiple pyknotic nuclei followed by karyorrhexis and eventually apoptosis. Similar results were found in this study too. Stickiness may result in pollen sterility depending on its intensity (Pagliarini 2000). In this study also, we have observed dark pink stained pollen grains which were considered to be fertile while those shrunken, colourless and empty ones were considered as sterile. Bridges and laggards are clastogenic abnormalities that are recorded in the present study. Occurrence of laggard chromosome may be due to unpaired chromosome at metaphase I or it can be due to lack of affinity between centromere and the spindle (Fisun & Rasgele 2009).

Acentric chromosome fragments were also observed that remain as laggards which may be due deletion and that may lead to the formation of micronuclei in successive generations. Stickiness between homologous chromosomes may also result in laggard formation (Gaulden 1987). Bridges are observed at both anaphase I and II. Acentric fragments may be associated with some bridges or paracentric inversion and is one of the common reasons associated with the formation of bridges (Haga 1953) but stickiness in chromosomes cannot be ignored as it can also lead to bridges. According to Saylor & Smith (1966) bridges might be due to chiasma failure to terminalize in a bivalent and thus chromosomes get protracted between the poles. Disturbed polarity and non-synchronization were also noted in this study at anaphase and telophase I/II respectively (Fig. 10). This could be due to spindle disturbance (Amer & Farah 1974). Triad formation with three microspores of different sizes could be a result of asynchrony in second meiotic division (Fig. 15). The disturbance or irregularities caused by deletion which occurred in meiosis I or II may lead to the formation of micronuclei at dyad and tetrad stages. The presence of laggards or fragments could be responsible for the formation of micronuclei at telophase II.

Chromosomal abnormalities are directly linked with pollen sterility (Mann 1978). It is the first sign of genetic toxicity of the treatment. The higher the extent of abnormalities the higher will be pollen sterility. This could be due to the cumulative effect of various meiotic aberrations. According to Ramanna (1974), deviation from karyokinesis and cytokinesis results in nonviable microspores as meiosis is prone to different types of cytological disturbances induced by chemical and physical mutagens (Swanson 1957). Pollen sterility could also be the result of interchanges of segments between nonhomologous chromosomes and presence of laggards, bridges, micronuclei and stickiness (Reddy & Rao 1982). Gaul (1970) concluded that chromosomal abnormalities are the major causes of pollen sterility induced by mutagens. The present study suggests that increased formation of micronuclei, bridges, laggards and most importantly, stickiness in chromosomes could be the reason of pollen sterility which was observed both in green and cured leaf extracts except the percentage of abnormality was less in meiocytes exposed to green leaf extracts. There are many reports about occupational hazard and GTS but the potential cytotoxic and genotoxic effects of green leaf extracts were observed for the first time as far as our knowledge goes.

Tobacco leaves in any form either cured or green tobacco are toxic and induce different types of abnormalities at both meiosis I and II. Percentage of abnormalities are directly proportional to the duration of exposure of both green and cured leaf extracts. So, from the above study it can be concluded that tobacco, both in its green form and cured form, induces meiotic abnormalities. However, green leaves show lesser abnormalities as compared to cured ones. But abnormalities shown by green leaves cannot be overlooked and

further research should be conducted on the use of green tobacco because our findings suggest that besides occupational hazard and GTS, it has mutagenic potential also. Increased abnormalities by cured form can be supported by the fact that curing process results in various biochemical changes in tobacco leaves which also increase the mutagenicity of the product.

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RESEARCH ARTICLE

EVALUATION OF ANTIBACTERIAL, PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITIES OF ENDOPHYTIC FUNGI FROM *CYMBIDIUM ALOIFOLIUM*, AN EPIPHYTIC ORCHID

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SUMMARY The endophytic fungi are versatile reservoirs of bioactive metabolites like antimicrobial and antioxidant agents. 43.33% of endophytic fungi associated with different parts of an orchid, *Cymbidium aloifolium* demonstrated antibacterial activity; 16.66% endophytic fungi, *Colletotrichum gloeosporioides* (CAR4), *Aspergillus terreus* (CAR14), *Alternaria alternata* (CAR15, CAL8) and *Fusarium oxysporum* (CAF1) exhibited broad spectrum antibacterial activity. The phytochemicals produced by these fungi were, steroids, tannins, flavonoids and alkaloids. The endophytic fungi exhibited 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity; the highest percentage of free radical scavenging activity (RSA) was in *Rhizoctonia* sp. (52.34 ± 2.5%) isolated from roots and the lowest was in *Aspergillus japonicus* (16.52 ± 1.14%). 50% of endophytic fungi showed total phenolic content (TPC) and *Rhizoctonia* sp. (CAR7) exhibited highest TPC of 4.099 ± 0.07 mg gallic acid/100 ml. TPC of endophytic fungal extracts were correlated with the DPPH scavenging activity.

Keywords: Endophytic fungi, antibacterial, phytochemical, antioxidant, phenolic content.

INTRODUCTION

The pathogenic microorganisms are the causative agents for the majority of infectious diseases worldwide. The bacterial infections are treated with antibiotics but, recently, bacteria are gaining resistance to antibiotics hence, it is the need of the hour to find new sources of drugs which have broad spectrum antimicrobial activity (Prakash et al. 2013). The past findings have proved that the plant-derived bioactive compounds have been successfully used in treatment of numerous infectious diseases and these compounds are also commercially used as potential drugs (Dias et al. 2012). The studies in the past few decades have focused on the endophytic fungi associated with medicinal plants including orchids which produce and mimic the host plant's secondary metabolites (Xing et al. 2011). The bioactive metabolites reported from endophytic fungi associated with various plants include alkaloids, steroids, flavonoids, terpenoids, saponins, tannins, phenolic compounds, quinines etc. The production of these bioactive endophytic fungal metabolites can be further enhanced using biotechnological tools (Tran et al. 2010, Jalgaonwala et al. 2017).

The adequate quantity of exogenous natural antioxidants helps to diminish the brunt of Reactive Oxygen Species (ROS) and there is an alluring stride to explore naturally existing antioxidants. The ROS such as superoxide anion and hydroxyl radical cause lipid peroxidation, protein denaturation, inflammation, ageing, cancer, neuron degenerative disorders and many more. The endophytic fungi associated within healthy plants are reported to be prospective sources of natural antioxidants. The augmented production of antioxidant compounds in endophytic fungi is due to numerous abiotic stress factors which increase reactive oxygen in host plant tissues (White & Torres 2010). The endophytic fungi surmount oxidative stress by production of antioxidants such as enzymes and phenolic compounds (Bacon & White 2016).

The copious studies have confirmed that numerous orchid species are hosts of immense endophytic fungal biodiversity. These endophytic fungi associated with orchids usually occur in distinct ecological niches and are sources of assortment of bioactive metabolites with immense prospective for new drug discovery (Shrestha et al. 2018). The endophytic fungi present within orchids also promote their seed germination; stimulate development and growth of protocorms, seedlings, adult plants and tubers by production of phytohormones, extracellular enzymes and other novel bioactive metabolites. The production of diverse metabolites by endophytic fungi depends on the host and its habitat (Hamayun et al. 2009).

The data on production of antibacterial, phytochemical and antioxidant metabolites from orchid endophytic fungi are skimpy hence, the present work was undertaken to evaluate the antibacterial, phytochemical and antioxidant activities from endophytic fungi associated with different parts of *Cymbidium aloifolium.*

MATERIALS AND METHODS

Isolation and screening for antibacterial activity of endophytic fungi

The healthy *C. aloifolium* orchid plants were collected from different regions of Western Ghats during flowering time and maintained in Orchidarium of the Department of Botany, Bangalore University, Bengaluru. The endophytic fungi inhabiting the roots, leaves and flowers of *C. aloifolium* was isolated on Potato Dextrose Agar (PDA) with 50 µg/ml tetracycline. The isolated endophytic fungal extracts (ethyl acetate extract dissolved in 10% dimethyl sulfoxide (DMSO) to obtain 10 mg/ml concentration) were tested for antimicrobial activity on Muller Hinton agar plates against human pathogenic bacteria, *Escherichia coli* (MTCC 443), *Klebsiella pneumoniae* (MTCC 109), *Bacillus subtilis* (MTCC 10619), *Staphylococcus aureus* (MTCC 3160) and *Pseudomonas aeruginosa* (MTCC 4996) by disc diffusion method (Wang et al. 2007). 10 µg/ml Ampicillin was used as positive control, and 10% DMSO was used as negative control. The zone of inhibition obtained was measured in mm.

Phytochemical analysis of ethyl acetate extracts of endophytic fungi

The endophytic fungi isolated from *C. aloifolium* exhibiting potent antibacterial activity were tested to determine the presence of phytochemicals like alkaloids, steroids, terpenoids, tannins, flavonoids and amino acids according to standard methods (Harborne 1998, Rangari 2002).

Antioxidant analysis of endophytic fungi

The cultivation and methanol extraction of endophytic fungi isolated from *C. aloifolium* was done following modified procedure of Srinivasan et al. (2010). The concentrated fungal extract (2 mg/ml) was evaluated for their antioxidant property.

The free radical-scavenging activity (RSA) was performed using 96 micro well plate and Bio Rad microplate reader following modified procedure of Fenglin et al. (2004). Trolox stock (0.4 mM) in methanol was used to prepare different concentrations of standard (Trolox). The reaction mixture consisted of 30 µL of fungal extract (2 mg/ml) or Trolox (Standard) and 270 µL of DPPH (0.1 mM/L) in methanol. The mixture was incubated for 30 min in dark and absorption measured at 490 nm. RSA of the standard and sample was calculated as a percentage of discoloration.

Percentage of free RSA = $[A_{DPPH} – A_S/A_{DPPH}] \times 100$

A_S - absorbance of solution containing the sample, A_{DPPH}- absorbance of DPPH solution.

The standard graph was plotted for different concentrations of Trolox versus RSA% and corresponding Trolox equivalent for the fungal extracts were determined.

Total phenolic content in endophytic fungal extracts

The total phenolic content (TPC) in endophytic fungal extracts was determined by modified method of Cai et al. (2004). 0.2 mL of methanol extract was oxidized with 2.5 ml of 10% Folin Ciocalteu phenol reagent and vortexed. 2 ml of 2% (w/v) sodium carbonate solution was added and vortexed vigorously. The same procedure was followed for standard gallic acid (5–50 µg/ml). All the tubes were incubated in dark at room temperature for 30 min and absorbance measured at 765 nm. The TPC in fungal extracts was expressed as gallic acid equivalent (GAE). i.e., mg GAE/100 ml culture media.

Statistical analysis

The experiments were performed in triplicates, means were analyzed statistically and Duncan multiple range test (DMRT) was carried out using SPSS software version 20; IBM, Armonk, NY, USA (Barrett et al. 2012).

OBSERVATIONS

Screening for antibacterial activity of endophytic fungi

The endophytic fungi isolated from *C. aloifolium* were screened against both Gram-positive (*B. subtilis*, *S. aureus*) and Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) (Table 1). The endophytic fungus, *Fusarium oxysporum* (CAF1) recorded higher zone of inhibition (12.33 mm) against *S. aureus.* The highest zone of inhibition against *B. subtilis* was in *Penicillium*

TABLE 1: Antibacterial activity of endophytic fungi isolated from *C. aloifolium* by disc diffusion method.

Fungal isolate	Plant part	Fungus	Zone of Inhibition in mm ± SD; n=3				
			E. coli	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*	*B. subtilis*
CAR 1	Root	*A. japonicus*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 2		*C. lunata*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 3		*Nigrospora* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 4		*C. gloeosporioides*	9.33 ± 0.57^{g}	7.33 ± 1.15^{c}	8.66 ± 0.57^{d}	9.66 ± 0.5^{7ef}	10.66 ± 1.52^{d}
CAR 5		*Trichoderma* sp.	0^{a}	0^{a}	0a	0^{a}	0^{a}
CAR 6		*Xylaria* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 7		*Rhizoctonia* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 8		*F. chlamydosporum*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 9		*P. citrinum*	0^{a}	0^{a}	0^{a}	7.66 ± 1.52^{bc}	10.66 ± 0.57^{d}
CAR 10		*Helminthosporium* sp	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 11		*Curvularia* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0a
CAR 12		*A. sydowii*	8.33 ± 0.57^{ef}	0^{a}	0^{a}	9.66 ± 0.57^{ef}	7.33 ± 1.15^{bc}
CAR 13		*Cladosporium* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAR 14		*A. terreus*	7.66 ± 1.15^{de}	6.33 ± 0.57^{b}	7.33 ± 1.15^{c}	7.66 ± 1.52^{bc}	6.66 ± 0.57^{b}
CAR 15		*A. alternata*	6.66 ± 1.15^{bc}	6.33 ± 1.52^{b}	6.33 ± 0.57^{b}	6.66 ± 1.52^{b}	10.66 ± 1.15^{d}
CAR 16		*F. oxysporum*	6.66 ± 0.57^{bc}	0^{a}	0^{a}	10.33 ± 1.15^{f}	6.33 ± 0.57^{b}
CAL1	Leaf	*P. chrysogenum*	0^{a}	0^{a}	6.33 ± 0.57^{b}	9.33 ± 0.57^{def}	10.33 ± 1.15^{d}
CAL2		*A. sydowii*	7.33 ± 0.57^{cd}	0^{a}	0^{a}	8.33 ± 0.57^{cd}	6.66 ± 0.57^{b}
CAL3		*Trichoderma* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAL4		*Rhizoctonia* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAL5		*C. lunata*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAL6		*P. citrinum*	0^{a}	0^{a}	0^{a}	8 ± 1^{c}	8.33 ± 1.08^{c}
CAL7		*C. truncatum*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAL8		*A. alternata*	6.33 ± 0.57^{b}	7.66 ± 0.57^{c}	6.66 ± 0.57^{bc}	10.33 ± 1.52^{f}	6.66 ± 1.15^{b}
CAL9		*Bipolaris* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAF1	Flower	*F. oxysporum*	8.66 ± 1.15^{fg}	8.66 ± 0.57^{d}	7.33 ± 1.52^{c}	12.33 ± 0.57^{g}	6.33 ± 0.57^{b}
CAF2		*T. rotundus*	7.66 ± 0.57^{de}	0^{a}	0^{a}	10.33 ± 1.52^{f}	10.66 ± 1.52^{d}
CAF3		*P. purpurogenum*	0^{a}	0^{a}	0^{a}	8.66 ± 0.57^{cde}	11.33 ± 0.57^{e}
CAF4		*Cladosporium* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
CAF5		*Cylindrocephalum* sp.	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
Positive control			11 ± 0.89^{h}	9 ± 1de	10 ± 0.93^{e}	17^{j} ± 0.9^{h}	15 ± 0.89^{e}

Positive control = Ampicillin 10 µg/mL; in each column, mean values followed by the same letter are not significantly different according to DMRT at $p < 0.05$.

TABLE 2: Phytochemical analysis of ethyl acetate extract of endophytic fungi from *C. aloifolium.*

Fungal isolate	Fungus	Phytochemical tests					
		Alkaloids	Steroids	Terpenoids	Tannins	Flavonoids	Amino acids
CAR 4	*C. gloeosporioides*	-	+	-	+	-	-
CAR 9	*P. citrinum*	-	-	-	-	+	-
CAR 12	*A. sydowii*	+	-	-	+	-	-
CAR 14	*A. terreus*	+	-	-	+	-	-
CAR 15	*A. alternata*	+	-	-	-	-	-
CAR 16	*F.oxysporum*	-	-	-	-	+	-
CAL1	*P. chrysogenum*	+	-	-	-	-	-
CAL2	*A. sydowii*	+	-	-	+	-	-
CAL6	*P. citrinum*	-	-	-	-	+	-
CAL8	*A. Alternata*	+	-	-	-	-	-
CAF1	*F. oxysporum*	-	-	-	-	+	-
CAF2	*T. rotundus*	+	-	-	-	+	-
CAF3	*P. purpurogenum*	+	-	-	-	+	-

+ Present, - Absent.

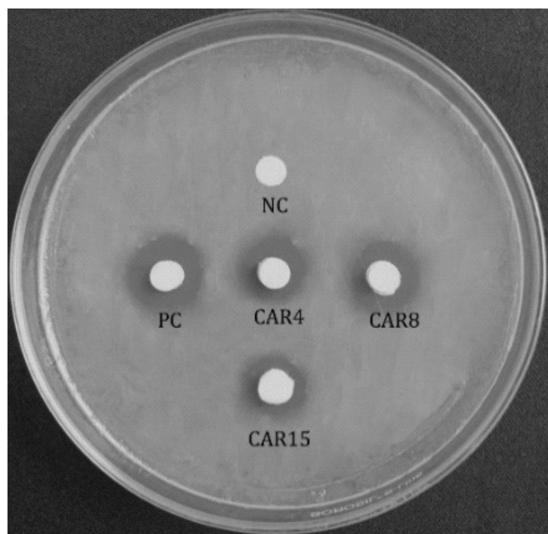

Fig. 1: Screening for antibacterial activity of endophytic fungi isolated from *C. aloifolium* against *E. coli*.

NC - Negative control, PC - Positive control, CAR4 - *C. gloeosporioides*, CAR8 - *F. chlamydosporum,* CAR15 - *A. alternata.*

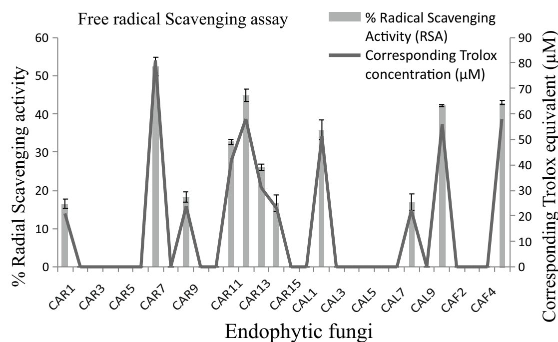

Fig. 2: Free radical scavenging activity of endophytic fungi from *C. aloifolium* and its corresponding Trolox equivalent (µM/ml).

purpurogenum- CAF3 (11.33 mm). The highest zone of inhibition against *E. coli* was recorded in *Colletotrichum gloeosporioides* - CAR4 (9.33 mm) (Fig.1); *K. pneumoniae* was recorded in *C. gloeosporioides* - CAR4 (8.66 mm); *P. aeruginosa* was recorded in *Fusarium oxysporum* - CAF1 (8.66 mm).

TABLE 3: Free radical scavenging activity of endophytic fungal extracts and its corresponding Trolox equivalent.

Fungal isolate	Plant part	Fungus	% RSA	TE µM/ml
CAR1	Root	*A. japonicus*	16.52 ± 1.14^{b}	21
CAR2		*C. lunata*	0^{a}	-
CAR3		*Nigrospora* sp.	0^{a}	-
CAR4		*C. gloeosporioides*	0^{a}	-
CAR5		*Trichoderma* sp.	0^{a}	-
CAR6		*Xylaria* sp.	0^{a}	-
CAR7		*Rhizoctonia* sp.	52.34 ± 2.5^{e}	81
CAR8		*F. chlamydosporum*	0^{a}	-
CAR9		*P. citrinum*	18.36 ± 1.29^{b}	24
CAR10		*Helminthosporium* sp.	0^{a}	-
CAR11		*Curvularia* sp.	0^{a}	-
CAR12		*A. sydowii*	32.65 ± 0.64^{cd}	42
CAR13		*Cladosporium* sp.	44.91 ± 1.69^{de}	59
CAR14		*A. terreus*	26.01 ± 0.75^{cd}	31
CAR15		*A.alternata*	16.66 ± 2.13^{b}	23
CAR16		*F. oxysporum*	0^{a}	-
CAL1	Leef	*P. chrysogenum*	0^{a}	-
CAL2		*A. sydowii*	35.87 ± 2.5^{cd}	51
CAL3		*Trichoderma* sp.	0^{a}	-
CAL4		*Rhizoctonia* sp.	0^{a}	-
CAL5		*C. lunata*	0^{a}	-
CAL6		*P.citrinum*	0^{a}	-
CAL7		*C.truncatum*	0^{a}	-
CAL8		*A. alternata*	16.94 ± 2.24^{b}	22
CAL9		*Bipolaris* sp.	0^{a}	-
CAF1	Flower	*F. oxysporum*	42.25 ± 0.27^{de}	56
CAF2		*T. rotundus*	0^{a}	-
CAF3		*P. purpurogenum*	0^{a}	-
CAF4		*Cladosporium* sp.	0^{a}	-
CAF5		*Cylindrocephalum* sp.	42.96 ± 0.63^{de}	57

In each column, mean values ± SD followed by the same letter are not significantly different according to DMRT at $p < 0.05$.

TABLE 4: Total phenolic content in endophytic fungi (extracts) isolated from *C. aloifolium.*

Fungal isolate	Plant part	Fungus	TPC (mg GAE/100 ml)
CAR1	Root	*Aspergillus japonicus*	0.854 ± 0.06[bcd]
CAR2		*Curvularia lunata*	0[a]
CAR3		*Nigrospora* sp.	0[a]
CAR4		*Colletotrichum gloeosporioides*	0.51 ± 0.13[abc]
CAR5		*Trichoderma* sp.	0[a]
CAR6		*Xylaria* sp.	0[a]
CAR7		*Rhizoctonia* sp.	4.099 ± 0.07[h]
CAR8		*Fusarium chlamydosporum*	1.15 ± 0.028[cd]
CAR9		*Penicillium citrinum*	1.06 ± 0.18[cd]
CAR10		*Helminthosporium* sp.	0[a]
CAR11		*Curvularia* sp.	0.29 ± 0.025[ab]
CAR12		*Aspergillus sydowii*	2.61 ± 0.14[e]
CAR13		*Cladosporium* sp.	3.63 ± 0.15[gh]
CAR14		*Aspergillus terreus*	2.48 ± 0.23[e]
CAR15		*Alternaria alternata*	0.92 ± 0.11[bcd]
CAR16		*Fusarium oxysporum*	0.78 ± 0.09[bcd]
CAL1	Leaf	*Penicillium chrysogenum*	0[a]
CAL2		*Aspergillus sydowii*	2.91 ± 0.19[ef]
CAL3		*Trichoderma* sp.	0[a]
CAL4		*Rhizoctonia* sp.	0[a]
CAL5		*Curvularia lunata*	0[a]
CAL6		*Penicillium citrinum*	0[a]
CAL7		*Colletotrichum truncatum*	0[a]
CAL8		*Alternaria alternata*	0[a]
CAL9		*Bipolaris* sp.	0[a]
CAF1	Flower	*Fusarium oxysporum*	1.25 ± 0.32[d]
CAF2		*Talaromyces rotundus*	0[a]
CAF3		*Penicillium purpurogenum*	0.81 ± 0.21[bcd]
CAF4		*Cladosporium* sp.	0[a]
CAF5		*Cylindrocephalum* sp.	3.25 ± 0.23[fg]

In each column, mean values ± SD followed by the same letter are not significantly different according to DMRT at $p < 0.05$.

Phytochemical analysis of ethyl acetate extract of endophytic fungi

The phytochemical analysis was carried out for endophytic fungi from *C. aloifolium* which exhibited antibacterial activity. The phytochemical analysis of *C. gloeosporioides* (CAR4) revealed the presence of steroids and tannins; *P. citrinum* (CAR9), *F. oxysporum* (CAR16), *P. citrinum* (CAL6), *F. oxysporum* (CAF1) revealed the presence of flavonoids, *Aspergillus sydowii* (CAR12), *A. terreus* (CAR14), *A. sydowii* (CAL2) revealed the presence of alkaloids and tannins; *A. alternata* (CAR15), *P. chrysogenum* (CAL1), *A. alternata* (CAL8) revealed the presence of alkaloids; *Talaromyces rotundus* (CAF2), *P. purpurogenum* (CAF3) revealed the presence of alkaloids and flavonoids (Table 2).

Antioxidant analysis of endophytic fungi

The endophytic fungi from *C. aloifolium* exhibiting scavenging activity were, *Rhizoctonia* sp. (52.34 ± 2.5%), *Cladosporium* sp. (44.91 ± 1.69%), *Cylindrocephalum* sp. (42.96 ± 0.63%), *F. oxysporum* (42.25 ± 0.27%), *A. sydowii* (35.87 ± 2.5%, 32.65 ± 0.64), *A. terreus* (26.01 ± 0.75%), *P. citrinum* (18.36 ± 1.29%), *Alternaria alternata* (16.94 ± 2.24%, 16.66 ± 2.13%) and *Aspergillus japonicus* (16.52 ± 1.14%) (Table 3, Fig. 2).

Determination of TPC in endophytic fungi

TPCs were recorded in methanol extracts of endophytic fungi isolated from *C. aloifolium* (Table 4); *Rhizoctonia* sp. (CAR7) exhibited highest TPC of 4.099 ± 0.07 mg gallic acid/100 ml followed by *Cladosporium* sp. (CAR13) TPC 3.63 ± 0.15 mg GAE/100 ml, *Cylindrocephalum* sp. (CAF5) TPC 3.25 ± 0.23 mg GAE/100 ml, *A. sydowii* (CAL2, CAR12) TPC 2.91 ± 0.19 and 2.61 ± 0.14 mg GAE/100 ml, *A. terreus* (CAR14) TPC 2.48 ± 0.23 mg GAE/100 ml, *F. oxysporum* (CAF1) TPC 1.25 ± 0.32 mg GAE/100 ml, *F. chlamydosporum* (CAR8) TPC 1.15 ± 0.028 mg GAE/100 ml and *P. citrinum* (CAR9) TPC 1.06 ± 0.18 mg GAE/100 ml. The endophytic fungi, *A. japonicus* (CAR1), *C. gloeosporioides* (CAR4), *Curvularia* sp. (CAR11), *A. alternata* (CAR15), *F. oxysporum* (CAR16) and *P. purpurogenum* (CAF3) recorded TPC of less than 1 mg GAE/100 ml.

DISCUSSION

The endophytic fungi inhabiting host plants are versatile reservoirs of numerous bioactive metabolites including antimicrobial agents which can be of potential use in modern medicine, industry and agriculture (Alurappa et al. 2018). In the present study, the antibacterial activities of the endophytic fungi associated with different parts of *C. aloifolium* were determined. The 43.33% of endophytic fungi demonstrated antibacterial activity by inhibiting more than one pathogenic bacterium tested. The present study agrees with the findings of Xing et al. (2011) who reported that 43.5% of endophytic fungal isolates associated with *Dendrobium thyrsiflorum* revealed antimicrobial activity.

16.66% endophytic fungi viz. *C. gloeosporioides* (CAR4), *A. terreus* (CAR14), *A. alternata* (CAR15, CAL8), and *F. oxysporum* (CAF1) exhibited broad spectrum antibacterial

activity. 43.33% endophytic fungi inhibited growth of Gram-positive bacteria (*S. aureus* and *B. subtilis*) tested, 30% isolates inhibited *E. coli*, 20% isolates inhibited *K. pneumoniae* and 16.66% isolates inhibited *P. aeruginosa*. The present findings are in accordance with Shi et al. (2018) who reported the antibacterial activity of endophytic fungus, *Fusarium* sp. obtained from the roots of *Dendrobium officinale*. The present findings also agree with those of Xing et al. (2011) who reported that *Fusarium* sp. isolated from *D. loddigessi* exhibited broad spectrum antimicrobial activity. Our findings agree with those of Deka & Jha (2018) who reported the antibacterial activity of endophytic fungi from leaves and barks of *Litsea cubeba* and also of Ding et al. (2010) who reported that the endophytic fungi of *Camptotheca acuminata* exhibited broad spectrum antimicrobial activity against pathogenic bacteria. The present study agrees with Lv et al. (2010) who reported that the endophytic fungi of *Saussurea involucrate* exhi-bited broad spectrum antimicrobial activity.

The phytochemical analysis was carried out for endophytic fungi which exhibited antibacterial activity. The phytochemical analysis of endophytic fungal extracts exhibiting antibacterial activity showed the presence of various chemical constituents which could have acted as the potent antimicrobial agent. In the present study, phytochemicals produced by endophytic fungi were, steroids, tannins, flavonoids and alkaloids. Our findings are in concurrence with those of Rampadarath et al. (2018) who reported that the endophytic fungi associated with *Jatropha* plant produced secondary metabolites like alkaloids, flavonoids, steroids and tannins and of Murthy et al. (2011) who reported that the endophytic fungi (extracts) isolated from *Lobelia nicotianifolia* showed the presence of various secondary metabolites including flavonoids. Further, the present study also agrees with the findings of Devi et al. (2012) who reported the presence of different phytochemicals such as alkaloids, flavonoids, and tannins from the endophytic fungi isolated from *Centella asiatica.* Doughari et al. (2007) and Cowan (1999) showed that the phytochemicals such as flavonoids and alkaloids act as antimicrobial agents. Rajagopal et al. (2018) reported that culture filtrates of *Curvularia lunata*, *Nigrospora oryzae*, *Chaetomium indicum* and *Pestalotiopsis microspora* from hydrophytic plants contained alkaloids, flavonoids, terpenoids, tannins and steroids. The endophytic fungi are reported previously to produce broad variety of bioactive secondary metabolites with unique structures including alkaloids, flavonoids, phenolic acids, steroids and quinones (Tan & Zou 2001).

The endophytic fungi are the potential amalgam of medicinally important compounds. The previous studies revealed that the microorganisms isolated from special ecosystems produce special activated metabolites (Stierle et al. 1993). In the present study, DPPH - a stable free radical with absorption at 490 nm was used to study the per cent radical scavenging activity of

endophytic fungal ethanol extracts. The anti-oxidants in the fungal extract donate proton to this free radical resulting in decreased absorption.

Endophytic fungi, a potential source of medicinal compounds, have attracted more and more attention in the last few years due to their ability to exhibit RSA. It is reported that micro-organisms isolated from special ecoenviron-mental conditions may produce metabolites like antioxidant compounds (Stierle et al. 1993). Hence, in the present study, endophytic fungi were screened for RSA. The highest per cent RSA was exhibited by *Rhizoctonia* sp. (CAR7) isolated from roots and the lowest per cent RSA was exhibited by *Aspergillus japonicus* (CAR1). The present study concurs with that of Srinivasan et al. (2010) who reported the DPPH scavenging activity of *Phyllosticta* sp., an endophytic fungus associated with the medicinal plant *Guazumato mentosa.*

The phenolic compounds are secondary metabolites produced by endophytic fungi which possess wide range of free radical scavenging and antioxidant activities due to presence of hydroxyl groups. TPC of endophytic fungal extracts were correlated with the DPPH scavenging activity. 50% of endophytic fungi of *C. aloifolium* screened produced TPC. Our findings are in accordance with Danagoudar et al. (2018) who reported high TPC in *Penicillium citrinum* isolated from *Tragia involucrate*, a medicinal plant. The phenolic acids have also been documented from the endophytic fungus *Annulohypoxylon stygium* BCR34024 (Cheng et al. 2014). In the present study, phenolic compounds were detected as reported by Das et al. (2018) in *Zingiber nimmoni*. According to them, varied phenolic profiles were attributed to the differences in their antioxidant capacities.

The present findings document the anti-bacterial and antioxidant activities of the various endophytic fungi associated with different parts of *C. aloifolium*, an epiphytic orchid plant. The production of different phytochemical constituents and the TPC are also reported. This helps to explore the potential bioactive metabolites associated with endophytic fungi of an orchid plant.

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