Original Article

Biological Activities of Endophytic Fungus *Cochliobolus* sp.AL24 Isolated from *Aerva lanata*. L

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ABSTRACT

Background: The plant *Aerva lanata* L belongs to the family Amaranthaceae and traditionally used in the treatment of rheumatism, kidney stones and malaria. In the present study endophytic fungus was isolated from *A.lanata* and screened of various extracts *in vitro* for their pharmacological potentials. **Objective:** To evaluate the antioxidant, antidiabetic and anti-inflammatory potentials of various extracts of endophytic fungus *Cochliobolus* sp from A. lanata. **Materials and Methods:** The fungus was identified using colony morphological and 18S rRNA sequencing technique. The 21 days grown endophytic fungus on potato dextrose broth was extracted sequentially with dichloromethane, butanol and methanol (marc) by cold extraction. The extracts were screened for qualitative and quantitative phytochemical analysis and evaluated their antioxidant activity by 2; 2- diphenyl-1-1-picryl hydrazyl radical scavenging assay. The antidiabetic activity was screened by alpha amylase assay. Also, the further evaluation of *in vitro* anti-inflammatory activity was done using heat induced egg albumin denaturation. **Results:** Based on colony morphology and molecular technique, the isolated endophytic fungus was identified as *Cochliobolus* sp. Preliminary phytochemical screening of various solvent extracts showed presence of alkaloids, phenolics, flavonoids and saponins. Methanolic extract exerts potent antioxidant, antidiabetic and anti-inflammatory potentials of 66.92 ± 0.86, 61.76 ± 1.07 and 62.51 ± 1.27% respectively. Moderately found in dichloromethane extract followed butanol extract. The quantitative determination of total phenol and flavonoid content was found high in the methanol extract of 400.3 ± 0.05 mg of gallic acid equivalent and 295.3 ± 0.05 mg of rutin equivalent/g of extract respectively followed dichloromethane and butanol extract. **Conclusion:** The present studies showed that methanolic extract of endophytic fungus *Cochliobolus* sp possess significant antioxidant, antidiabetic and anti-inflammatory potentials.

Keywords: *Aerva lanata,* Endophyte fungus, Antidiabetic, Anti-inflammatory, Antioxidant.

INTRODUCTION

Natural products are the important resources for the discovery of new drugs, medicinal plants and fungal endophytes which have an effective and wide range of applications in medicine, agriculture, cosmetics, and industry.[1] The novel bioactive compounds with a remarkable chemical diversity are originated in the huge number of species of plants, animals, marine organisms and microorganisms.[2] Endophytes are microbes (fungi and bacteria) that live symbiotically in the host plant, without affecting the host and their ability to synthesize various useful/ health beneficial bioactive compounds and these compounds may involve in defense mechanisms against phytopathogens.[3] Taxolis an anticancer drug using commercially was also produced by the endophytic fungus *Taxomyces andreanae* from the plant *Taxus* sp. which can involve in defense mechanism against plant pathogenic fungi.[4] Endophytes are a source of wide range of bioactive secondary metabolites, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones and many others.[5] Such bioactive metabolites find

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extensive used in the field of agrochemicals, food and pharmaceutical industries.[6]

Aerva lanata.L (family: Amaranthaceae), synonym *A. sanguinolenta*. Blume, is also known as Paashaanabheda found throughout the tropical India as a common weed in field and waste places. Its habitat in warmer parts of India ascending to 1000 m.[7] It has small and simple leaves which are 1.3-3.5 cm long and 0.8-2.5 cm wide, shiny white, red at the base and has two lobes. A.lanata flowers during the month of May and October. The flowers are tiny about 2.5 mm long with creamy white color; grow in clusters near the axis of stems and flower has no honey or fragrance.[8] The Plant is used for treating anticalculus, diuretic, demulcent, anthelmintic, antidiarrhoeal, anticholera, bechic; leaves in hepatitis and root in strangury. The catarrh of the bladder is treated by the plant of a decoction. The flowers and roots were used for headache.[7] and reports on diuretic,[9] antidiabetic,[10] antimicrobial,[11] hepatoprotective.[12] and anti-diarrhoeal potentials.[13] The plant was reported to have alpha-amyrin, campesterol, beta-sitosterol, palmitate, chrysin and flavonoid glucosides from heartwood.[7] The present study was to investigate the anti-oxidant, anti-diabetic and anti-inflammatory potentials various extracts of endophytic fungal from A. lanata.

MATERIALS AND METHODS

Sample Collection

Fresh, healthy leaves; stem and root of *A. lanata* were collected from Vellore district, Tamil Nadu in the month of January 2016. The samples were collected in sterile bags and were at 4ºC for further use.

Isolation of endophytic fungi

The collected samples of *A. lanata* were washed methodically with tap water to remove dirt spots and surface was sterilized with 4% sodium hypochlorite solution. Then samples were aseptically cut into small fragments and placed on Petri plate containing potato dextrose agar (PDA) and plates were incubated at 28ºC for 4 days. The pure cultures were documented and preserved for further research.[14]

Cultivation of endophytic fungi

The purified fungal culture was cultivated on potato dextrose broth and incubated at room temperature for 21 days under static conditions.[15] the broth culture was filtered to separate the filtrate and mycelia. The filtrate was extracted three times by shaking with an equal volume of different solvents petroleum ether, dichloromethane (DCM), ethyl acetate and butanol. The mycelial mat was extracted with methanol. The solvent extracts were concentrated using a rotary vacuum evaporator and then dried extracts were used directly to assess the *in vitro* assays.

Molecular identification of endophytic fungi

The isolated strains were identified based on the molecular techniques. PCR reactions for sequencing were carried out in a PTC-100™ thermal cycler with universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3') The amplified fragment includes ITS1, 5.8S and the ITS2 of rDNA. Amplification was performed in a 50 µl reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X 100, 2 mM MgCl2, 0.2mM dNTPs, 0.4 µM primers, 0.5 unit of Taq polymerase, and 2µl of fungal genomic DNA. The thermal cycling program was as follows: 2 min initial denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s primer annealing at 55°C, and 45°s extension at 72°C, and 5 min at 72°C for a final extension. The PCR product was purified with High Pure PCR Product Purification Kit. Direct DNA sequencing was performed using primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS5 on an ABI 3100 automated sequencer following the manufacturer's instructions. The consensus sequences were deposited in the GenBank.[16]

Phytochemical screening

Various fungal extracts were subjected for phytochemical screening to identify the nature of the secondary metabolite present in it.[17]

Quantification of total phenolics and flavonoid contents

Total phenolic content in extracts was determined with Folin-Ciocalteut reagent using gallic acid as a standard.[18] briefly, 0.1 ml sample solution in methanol (1.0 mg/ml) was mixed with 1.5 ml of Folin-Ciocalteu reagent and then 1.5 ml of 6% sodium bicarbonate solution was added to the mixture and it was incubated for 90 min at room temperature. The absorbance was read at 725 nm in a UV-spectrophotometer. The results were expressed as milligrams of gallic acid equivalent/g sample. The flavonoid content in fungal extracts was determined using aluminum chloride method colorimetric assay.[19] Total flavonoid content was expressed as milligrams of rutin equivalents/g sample.

DPPH free radical scavenging assay

The radical scavenging activity of the various extracts of *Cochliobolus* sp was evaluated using 2, 2-diphenyl-1-pycriylhydrazyl (DPPH) radical. DPPH is a stable free

radical which can be reduced to a yellow colored product, diphenyl pycriyl hydrazine when a substrate donates a hydrogen atom.[20] the inhibition percentage of DPPH radical was calculated as given formula:

$$\text{Inhibition of DPPH radical \%} = \left(\frac{\text{Abs of control} - \text{Abs of test}}{\text{Abs of control}}\right) \times 100$$

Determination of α –amylase inhibitory activity

The 0.1 ml of the fungal extract along with 0.1 ml of 0.02 mol/L sodium phosphate buffer (pH 6.9) and 0.1 ml α-amylase solution (1mg/ml) was mixed and pre-incubated at 25°C for 10 min. Then, 0.1 ml of 1% starch solution was added and incubated at 25°C for 30 min and the reaction was stopped by the addition of 1.0 ml dinitrosalicylic acid reagent. The test tubes were incubated and then kept in a boiling water bath for 5 min and cooled to room temperature. The reaction mixtures were then diluted 10-fold times with distilled water and the absorbance was measured at 540 nm. The readings were compared with the control (the sample was replaced by buffer) and α-amylase inhibition activity (%) was calculated.[21]

$$\text{Inhibition of } \alpha \text{ amylase \%} = \left(\frac{\text{Abs of control} - \text{Abs of test}}{\text{Abs of control}}\right) \times 100$$

Inhibition of heat-induced albumin denaturation

We measured the capability of various fungal extracts to inhibit heat-induced albumin denaturation.[22] The reaction mixture consisted of 2 ml of various concentration (50, 100, 150 mg/ml) of each extract and the same volume of the vehicle alone (DMSO), 2.8 ml of phosphate buffer 0.2 M (pH 7.4) and 0.2 ml of egg albumin (from fresh hen's egg). The mixture was incubated at 37°C for 20 min and then heated to 51°C for other 20 min. After cooling, the turbidity of the samples was measured by reading the absorbance at 660 nm. Aspirin was used as the reference drug and the percentage inhibition of protein denaturation was calculated by using the following formula.

$$\text{Inhibition of denaturation \%} = \left(\frac{\text{Abs of control} - \text{Abs of test}}{\text{Abs of control}}\right) \times 100$$

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FT-IR analysis was performed to investigate the different functional groups present in the methanol extract of *Cochliobolus* sp. FT-IR analysis was performed on Nicolet FT-IR in 400-4000cm^{-1}.[23]

Statistical analysis

All the experiments were done in triplicates (n=3) and the results were expressed as Mean± SD. The data were statistically analyzed using one-way ANOVA. The 'P value' was found to be <0.05, which is significant.

RESULTS

The endophytic fungus *Cochliobolus* sp was isolated from the healthy leaves of the *A. lanata* and it was identified based on colony morphology, microscopic and molecular studies were analyzed by 18s rRNA analysis. *Cochliobolus* sp. is dark brown to black, unilocular with a globose body and a long or short cylindrical ostiolate neck. Hyaline to brown sterile hyphae and conidiophores often occur on the ascomata and less so on the neck. Figure 1.

Molecular analysis of the fungal isolate was based on ITS rDNA. The consensus sequences showed a 100% match with *Cochliobolus* sp. (GenBank accession number KY800380). The pictorial representation of the fungus is shown in Figure 1. A phylogenetic relationship was established through alignment and cladistic analysis of homologous nucleotide sequences and shown in Figure 2.

Phytochemical screening

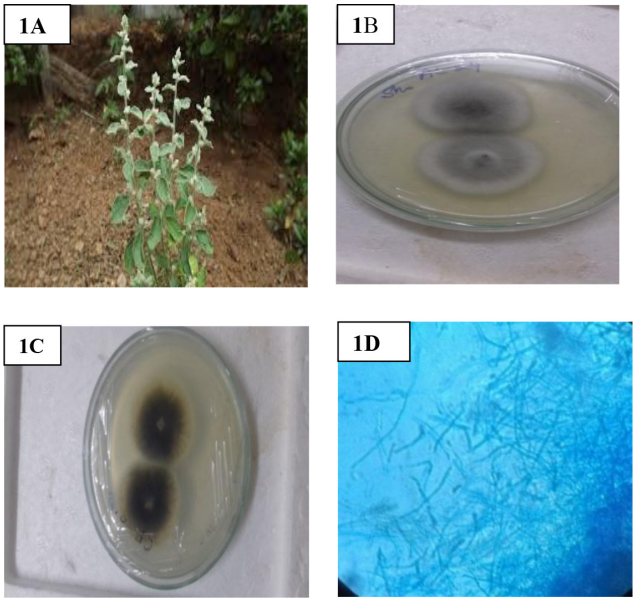

Figure 1: A] The host *Aerva lanata* plant B] Colony of pure culture of endophytic *Cochliobolus* sp. C] Reverse position of pure culture plate D] Microscopic fructification of *Cochliobolus* sp.

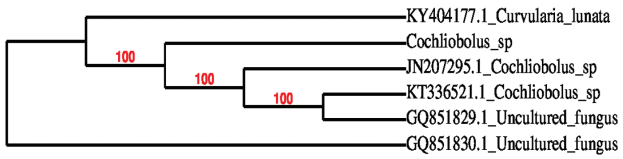

Figure 2: Phylogenetic tree based on the sequence homologies of their ITS sequences.

Various solvent extracts of endophytic fungi *Cochliobolus sp* was screened for the presence of phytochemicals and the results are depicted in Table 1.

Determination of total phenolic and flavonoid content

The methanol extract possesses maximum phenolic content of 400.3±0.05 mg/g of extract compared to other extracts followed DCM, butanol and their results were shown in Table 2. The content of flavonoid was expressed in terms of rutin equivalent (mg of RE/g) of extract (the standard curve equation: y =0.0292 x–0.0972, R^2=0.9892). The methanol extract also holds the highest flavonoid content of 295.3±0.05 mg RE/g, butanol and DCM extract both showed similar flavonoid content. The concentration of flavonoids in the extract was depended on the polarity of solvents used in the extraction.

DPPH free radical scavenging assay

DPPH has been a widely used method for free radical-scavenging activity due to its ease and sensitivity. In the present study, all extracts showed to be effective scavengers against DPPH radical compared to ascorbic acid with dose-dependent manner (Table 3). The methanol extract exhibits the maximum activity of 66.92±0.86%, while the minimum scavenger was the DCM extract of 42.29±0.71%.

Alpha amylase inhibition assay

Diabetes affects the major organ functions due to the high concentration of blood sugar.25 The result of alpha-amylase inhibition activity of various extracts of *Cochliobolus* sp was shown in Table 3 Above results revealed that all the extract possesses significant inhibition of alpha-amylase in a dose-dependent manner (50 to 150µg/ml) when compared with standard acarbose. The methanol extract exhibit significant inhibition (61.76±1.07%) compared to other extracts.

Inhibition of heat-induced albumin denaturation

In the present study, the *in-vitro* anti-inflammatory activity of fungal extracts performed by heat-induced egg albumin denaturation method and the results were represented in Table 3. The methanol extract shows the highest inhibition of 62.51±1.27%, followed butanol extract 56.74±1.03% and lowest found in DCM extract of 46.24±1.06%. The standard aspirin showed maximum inhibition of 85.43±0.005%

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FT-IR spectrum of methanol extract of *Cochliobolus* sp shows distinct peaks at 3331, 2943, 2831,1710,1438,1369,1228,1112,1026,646 and 536 cm^{-1} (Figure 3). The broad and strong peak at 3331cm^{-1} suggests (O-H) bond in stretching vibration mode and peak at 2943 and 2831 cm^{-1} indicates C-H (aliphatic)

Table 1: Phytochemical screening of various extract of *Aerva lanata*.

Phytochemicals Tests	Dichloromethane	Butanol	Methanol (Marc)
Alkaloids -Mayer's test	+	-	+
Phenols -Lead acetate test	+	-	+
Tannin -$Fecl_3$ test	+	+	-
Flavonoid –NaOH	-	+	+
Protein -Biuret test	-	-	+
Saponin -Froth forming test	+	-	+

Table 2: Total phenolics and flavonoid contents of endophytic fungi *Cochliobolus sp*.

Solvent Extract	Total Phenolics(mg GAE/g extract)	Flavonoids (mg RE/g extract)
Dichloromethane	300.3±0.05	260.3±0.05
Butanol	319.6±0.05	278.3±0.05
Methanol	400.3±0.05	295.3±0.05

Table 3: Inhibition percentage of DPPH, α-amylase, Egg albumin denaturation of endophytic fungus *Cochliobolus sp.*

Assays	Solvent Extract	Concentrations(µg/ml)		
		50	100	150
DPPH	Dichloromethane	42.29±0.71	46.22±0.34	48.22±0.58
	Butanol	46.52±0.55	48.36±0.049	49.32±0.57
	Methanol	56.28±1.077	66.04±0.78	66.92±0.86
	Ascorbic acid	90.08±0.005	92.65±0.005	93.12±0.005
α amylase	Dichloromethane	46.59±0.53	52.45±1.10	52.96±0.56
	Butanol	41.00±0.47	44.91±0.14	46.54±0.60
	Methanol	52.39±0.57	61.76±1.07	61.42±0.43
	Acarbose	63.34±0.005	74.01±0.005	77.24±0.005
Egg albumin denaturation	Dichloromethane	46.24±1.06	56.34±1.26	57.86±0.22
	Butanol	51.84±1.06	53.69±1.18	56.74±1.03
	Methanol	51.95±0.76	62.03±0.37	62.51±1.27
	Aspirin	78.35±0.005	81.84±0.005	85.43±0.005

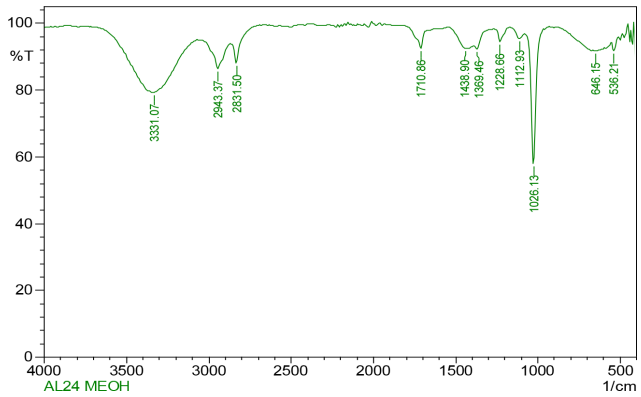

Figure 3: FT-IR results for methanol extract of *Cochliobolus* sp.

alkane stretch symmetry in stretching mode. The wave numbers 1710 cm^{-1}showed the presence of C=O (carbonyl) stretch. The wave number 1438 cm-1 and 1369cm^{-1} confirmed the presence of C=C (aromatic) alkene stretch and C=C (aromatic); C-H alkane bend respectively.[22] The peak at 1228, 1112 and 1026 cm^{-1} suggested the presence of C-O stretch. The weak peak at 646 and 536cm^{-1} represented the presence of =C-H alkene bend in the spectrum.

DISCUSSION

Endophytic fungi are considered to be a dependable source of novel natural compounds with a high level of biodiversity and may also yield several compounds of therapeutic importance which is currently attracting scientific investigations worldwide.[26] In nature; plants seem to be in a close interaction with endophytic fungi.[27] *A. lanata* leaves are used to treat antimalarial, urinary troubles, rheumatoid disease and also used for arresting hemorrhage during pregnancy, headache and skin diseases.[28] The endophytic fungus isolated from the plant *A. lanata* has been recognized as *Cochliobolus* sp based on microscopic and molecular studies. There are previous reports of *Cochliobolus* species isolated from an endophytic fungus from *Nerium oleander*.[29]

In this study, investigated the phytochemical screening, total phenolic content, flavonoid content, antioxidant, antidiabetic and anti-inflammatory potentials of a various extracts of endophytic fungus from *A. lanata* leaves by *in vitro* methods were investigated.

The preliminary phytochemical analysis of all extracts of endophytic fungus confirms the presence of alkaloids, flavonoids, phenols, saponins. The highest levels of total phenolic (400.3±0.05 mg GAE/g) and flavonoid contents (295.3±0.05 mg RE/g) were found in methanol extract while dichloromethane extract shows the lowest 300.3±0.05 mg GAE/g and 260.3±0.05 mg RE/g respectively. In previous report, the endophytic fungi of *Cochliobolus* sp. was isolated from the plant *Costus spiralis* (Jacq.) and the hydroethanolic extract of endophytic fungi possess rich total phenolic content of 528mg of TAE/ g of extract.[30] This data is slightly increased than the present investigation and it denotes that properties of solvent are taken in notice.

DPPH free radical scavenging assay is a basic and most widely used assay and considered most accurate screening method used to evaluate the antioxidant activity of samples.[28] Methanol extract shows highest DPPH free radical scavenging activity of 66.92±0.86% compared to standard ascorbic acid (93.12±0.005%).

Pancreatic α-amylase, the major enzyme involved in the conversion of starch to glucose which is absorbed into the blood circulation. Inhibition of α-amylase would reduce the liberation of glucose. The methanol extract exhibits significant α-amylase inhibition of 61.76±1.07% and earlier studies reported phenolic compounds have the capacity to inhibit the α-amylase enzyme. Therefore, the α-amylase inhibition and antioxidant potential of the methanol extract might be due to the occurrence of phenolic content.[20] the significant percentage inhibition of heat- induced protein denaturation was exhibited in methanol extract which shows strong anti-inflammatory potential when compared to standard aspirin.

The FTIR spectrum was used to analysis functional group of the bio active compounds present in the extract based on the obtained peak value.[24] the result of this study concludes that endophytic fungi may help as a source of rich phenolic bioactive compounds.

CONCLUSION

The results from this study clearly showed that methanolic extract of *Cochliobolus* sp possess significant antioxidant, antidiabetic and anti-inflammatory potentials. FTIR indicates to presence of aliphatic, aromatic and carboxyl groups in the methanolic extract may be responsible for biological activities. The FTIR analysis of methanolic extract validated the peak responsible for the presence of functional group OH (alcohol), C-H (aliphatic), C=O (carbonyl), C=C (aromatic) and =C-H (alkene). All the functional group presence in the methanolic extract comprises that extract may be phenolic rich in nature. The OH functional group has got the ability of forming hydrogen bonding capacity and responsible for the higher potential of antioxidant, anti-inflammatory activity of methanolic extract. The result of this study concludes that methanolic extract of endophytic fungi may help as a source of rich phenolic bioactive compounds. Furthermore, it is necessary to isolate and identify the bioactive compound present in the *Cochliobolus* sp. responsible for antioxidant, antidiabetic and anti-inflammatory that could be a better compound for designing a drug in the pharmaceutical applications.

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CONFLICT OF INTEREST

Authors declare there is no conflict of interest.

ABBREVIATIONS USED

GAE: Gallic Acid Equivalence; **DCM:** Dichloromethane; **DMSO:** Dimethyl Sulfoxide; **DPPH:** 2; 2- Diphenyl-1-1- Picryl Hydrazyl; **dNTP:** Deoxy Nucleotide; **FTIR:** Fourier Transform Infrared; **ITS:** Internal Transcribed Spacer; **PDA:** Potato Dextrose Agar; **PCR:** Polymerase Chain Reaction; **rRNA:** Ribosomal Ribonucleic Acid; **SD:** Standard Deviation; **TAE:** Tannic Acid Equivalence.

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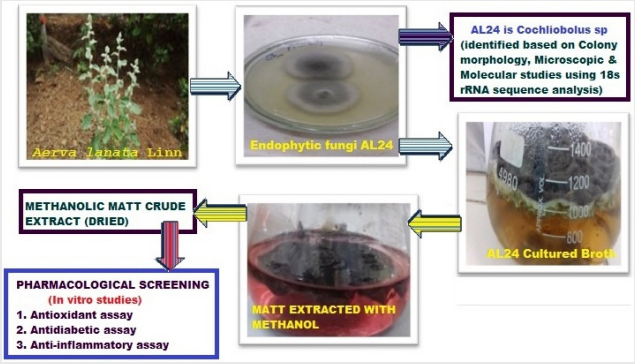
PICTORIAL ABSTRACT



SUMMARY

- Endophytic fungus (AL24) was isolated from the plant Aerva lanata Linn
- AL24 is identified as Cochliobolus sp. based on colony morphology, microscopic and molecular studies using 18s rRNA sequence analysis.
- Three weeks grown fungal cultured broth was filtered and to the fungal matt (mycelium) was extracted with the methanol solvent
- Methanolic extract was filtered from the matt and dried with using rotary evaporator
- Dried methanolic extract was screened for the antioxidant, antidiabetic and anti-inflammatory potentials
- FT-IR of methanolic extract revealed the presence of phenolic compounds

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