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Potential Activity of Fungal Endophyte Lasiodiplodia Irregularis Isolated from Guiladina Bonduc as a Source of Antimicrobial Compound

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Abstract

The endophytes, are the microorganism which inhabit in distinct parts of the plant without showing any symptom. Fungal endophytes are treasury of therapeutically significant bioactive compounds. Guilandina bonduc is a divine tree, and important indigenous medicinal plant. It has been utilized in medicinal systems of Ayurveda, Siddha and Unani for treating several diseases. The aim focuses on isolation of fungal endophyte Lasiodiplodia irregularis from the plant part of Guilandina bonduc. It is a store house of endophytes, that produces magnificent therapeutic compounds. Thin layer chromatography (TLC) and Gas Chromatography-Mass Spectrometry (GCMS) were used to determine the chemical composition of fungal extract of endophytes. Our analysis of fungal extract through TLC and GC-MS shows the presence of 25 different type of compounds. The qualitative survey of methanolic solution of endophytic fungus confirms for terpenoids, steroids, carbohydrates alkaloids, flavonoids, tannins, and amino acids. Antimicrobial activity performed against Gram-negative and Gram-positive bacteria by disc diffusion method inhibited the growth of bacteria.

Keywords: Endophytes, Gas Chromatography-Mass Spectrometry GC-MS, Guilandina bonduc, Lasiodiplodia irregularis, Secondary metabolites, TLC (Thin Layer Chromatography).

Introduction

In recent time, fungal endophytes have been emerged as promising sources of natural compounds for drug discovery and therapeutic applications. Bacon and White, 2000 endophytes are popular entities found in the cells of plants, in hole lifespan except infecting the host. Majority of the endophytic fungi belong to Ascomycota and colonize the intercellular spaces of the plant without any visual symptoms (Correa et al., 2014). Among them, fungal endophytes have increased attention to establish mutualistic associations with host plants and synthesize a range of bioactive compounds. These metabolites not only protect the host against environmental stress and pathogens but also exhibit significant pharmacological potential, including antimicrobial, antioxidant, and anticancer properties (Gunatilaka, 2006., Tan & Zou, 2001., Cui, 2015., Yougen, 2015). Endophytic fungi are capable of producing a wide range of bioactive

compounds. Through accumulation of secondary metabolites, the endophytic fungi reduce the damage from the pathogens to host plant (Cabezas et al., 2012). Many of the bioactive compounds produced by these fungi are applicable in environment, agriculture, food and pharmaceutical industries (Deshmukh et al., 2015; Suryanarayanan et al., 2009; Kharwar et al., 2011).

Guilandina bonduc (syn. Caesalpinia bonduc), belongs to Fabaceae family, is a well-documented medicinal plant of high therapeutic value. Commonly known as Bonduc-nut, Fever-nut, or Physic-nut, it has been extensively utilized in the traditional systems of Ayurveda, Siddha, and Unani medicine (Omkar et al., 2006). Different parts of this plant exhibit a wide range of medicinal properties. Seeds are employed in the treatment of vomiting, leprosy, blood disorders, and diabetes, and are reported to possess hypoglycemic activity (Goyal et al., 1999). Leaves are traditionally used against infections, liver disorders, smallpox, and intestinal worms (Agrawal & Pal, 2013). Roots, either powdered with honey or decocted, are administered for leucorrhoea, fever, diabetes, and menstrual regulation (Kirtikar & Basu, 1993; Sasmal et al., 2007). Additionally, they act as expectorants and remedies for ophthalmic problems. The bark is valued as a tonic for jaundice and spleen-related ailments (Narendhirakannan, 2010; Sah & Verma, 2012; Desai et al., 2016).

Given the extensive medicinal importance of G. bonduc, recent research has shifted toward its associated endophytic fungi. It is hypothesized that endophytes may produces bioactive secondary metabolites identical to those synthesized by their host plant, thereby serving as alternative and sustainable sources of therapeutic agents. Despite the vast pharmacological potential, endophytic fungi associated with G. bonduc remain largely unexplored, with only limited studies reporting their diversity and bioactive properties.

The research emphasizes its mass culture, methanolic extraction, preliminary qualitative screening of antimicrobial activity, secondary metabolites, against Gram-negative and Gram-positive bacteria, and characterization of bioactive compound through TLC and GC-MS. This work aims to highlight the action of L. irregularis as a novel source of pharmacologically significant compounds for drugs development.

Materials and Methods

Sample Collection

The healthy and fresh twigs and leaf of Guilandina bonduc, a traditionally and pharmacologically significant plant, selected from botanical garden of CMP Degree College, University of Allahabad, Prayagraj, Uttar Pradesh (25°26′09″N; 81°50′47″E).

Surface Sterilization and Inoculation

Plant samples were thoroughly rinsed in tap water and after that sterile distilled water for adhered debris removal. Surface sterilization was performed sequentially with 0.01% $HgCl_2$ (1 minute), and 70% ethanol (2 minute) and again with sterile distilled water. The samples were blot dried aseptically, trimmed at the edges, and cut into ~0.5 cm fragments using a sterile scalpel.

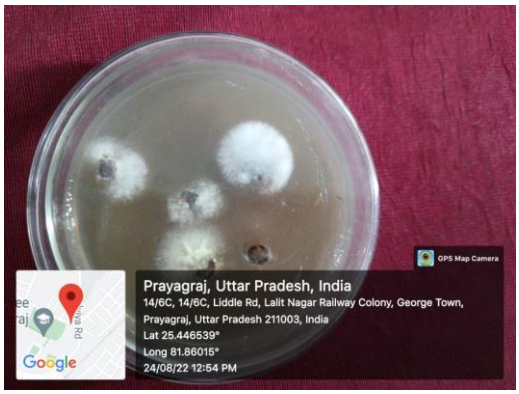
Explants were aseptically inoculated onto (PDA) potato dextrose agar plates and make tight with parafilm. Plates were kept for incubation at the temperature of (25 ± 2 °C) for 7–10 days and watch it carefully for growth of fungal hyphae. Emerging fungus hyphae were cultured again for obtaining pure cultures (Dos Reis et al., 2022).

Identification by morphology of endophytic fungi

Fungal isolates were initially characterized by colony morphology (color, texture, margin, and sporulation). Microscopic features were studied using lactophenol cotton blue staining under low and high power of microscope (Mita et al., 2021). Morphological identification was performed following standard fungal identification manuals (Harborne, 1998). The pure and fresh mycelia were cultured again and cultured it in PDA media slant at 4°C for next experiments. For mass cultivation, actively growing mycelia, inoculation was done on 250 ml Erlenmeyer flask in100 ml sterilized (PDB) i.e. potato dextrose broth and incubated it at 26 ± 2°C for 10–20 days.

Endophytic fungus growth on PDA media

Culture plates of endophytic fungus



Preliminary Phytochemical Screening

Methanolic extracts of L. irregularis was used further for better phytochemical assays following standard protocols to detect secondary metabolites including alkaloids, flavonoids, tannins, saponins, terpenoids, triterpenoids, steroids, carbohydrates, cardiac glycosides, and amino acids (Galindo-Solis et al., 2022).

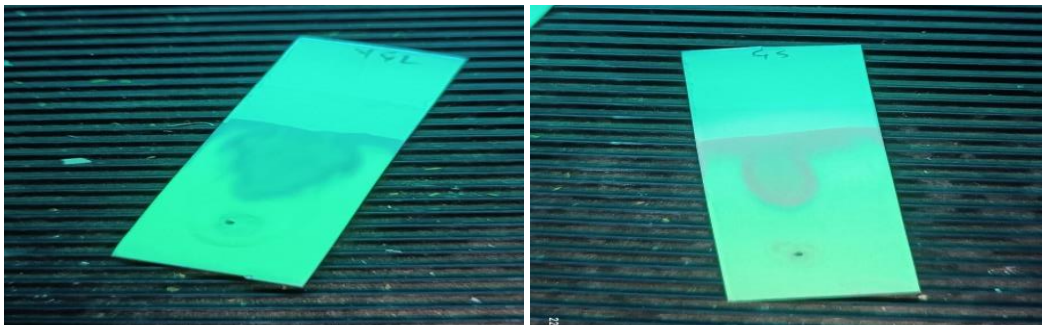
Fig. Crude extract culture of fungal endophyte L. irregularis

Thin Layer Chromatoghraphy (TLC)

Crude extract of test microbes and fractions were chemically screened and analysed using thin layer chromatography (TLC). On the glass plates measuring 10 x 25 cm^2 preparatory TLC plates were made using Merk's Silica gel 60 F_{254}. By applying a 7:1 solvent mix of Chloroform to Methanol, and the fungal extract was drop down to see the running on TLC plate. (Kowalska et al., 2022)

fig.1 TLC image of crude extract of fungus *Guilandina*

Fig. 2



GC MS analysis

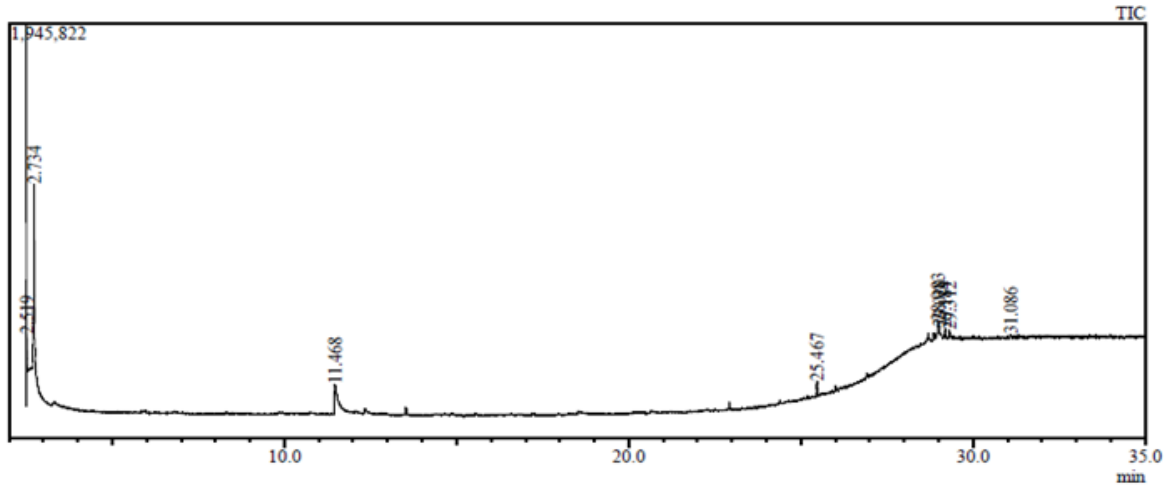
GC MS analysis of L. irregularis endophytic fungal extract was executed utilizing GCMS with AOC-20i auto injector and model No.-GCMS-TQ8040. The apparatus is utilized for mass spectrometer GC-MS,

furnished with Elite 1, capillary column fused with Silica. For GC MS exploration, the electron ionisation device were maintained with ionising potential of 70eV. Helium (He) gas of purity 99.99 % was carrier gas, by the regular flow of 1 ml/minute and injection capacity of 2µl (10:1 split ratio); injected temperature of 250°C, the ion source temperature of 230°C. The temperature of oven was regulated at 1100°C (isothermal for 2 minutes) to 200°C after if 50ºC per minute to 280ºC crowning by 9 minutes of isothermal temperature of 280^{0}C the mass of spectra was acquired around 70 eV, scanning period of 0.5 seconds the parts extending to 45 - 450 Da, complete Gas Chromatography operation period taken to 45.67 minutes.

The relative percent quantity of every constituent were determined with comparative analysis of its middle peak range to overall area. For managing mass of spectra as well as chromatogram a turbo mass was employed (Farhat et al., 2022). The GC and MS were done by methanolic extract of endophytic fungi L. irregularis. The analysis discloses important compounds corresponding to detectable peaks.

Peak	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	2.519	197710	6.35	260655	16.09	4-Vinylcyclohexene diepoxide (isomer 2)	41.10
2	2.734	1467457	47.16	892346	55.07	Silanediol, dimethyl-	77.05
3	11.468	520918	16.74	124408	7.68	2-Undecene, 4,5-dimethyl-, [R*,R*-(E)]-	69.10
4	25.467	115655	3.72	69339	4.28	Squalene	69.10
5	28.983	234431	7.53	88697	5.47	Khusimyl methyl ether	119.10
6	29.020	129205	4.15	59761	3.69	Selenophene-2-carboxylic acid, trimethylsilyl	189.15
7	29.184	132065	4.24	48547	3.00	9,19-Cyclolanost-24-en-3-ol, acetate, (3.beta)	408.35
8	29.312	128860	4.14	44348	2.74	(3S,6aR,6bR,8aS,12S,14bR)-4,4,6a,6b,8a,11,	189.15
9	31.086	103768	3.33	22308	1.38	1H,2H,3H,4H-Pyrido[3,4-b] pyrazine, TMS (is	207.00
10	39.895	81651	2.62	9970	0.62	(1R,2S,8As)-8-oxo-1-carboxymethyl-1,2,5,5-t	207.00
		3111720	100.00	1620379	100.00		

Fig. Guilandina leaf (young leaf crude on PDB)



Peak Report TIC

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(A)

Fig. GCMS result of L. irregularis.

Several bioactive compounds were identified based on their retention time, area, molecular formula, molecular weight in the form of compounds named as: 2-Nonynoic acid, Silanediol, dimethyl- , Phenazine, 3-Heptanol, 2,4-dimethyl- , Squalene, 4- Vinylcyclohexene diepoxide (isomer 2), Silanediol, dimethyl-,[R*,R*-(E)]-, Squalene,Khusimyl methyl ether, Solenophene-2-carboxylicacid, trimethylsilyl, 9,19-Cyclolanost-24-en-3-ol, acetate, (3.beta), (3S,6aR,6bR,8aS,12S,14bR)-4,4,6a,6b,8a,11,1H,2H,3H,4H-Pyridol[3,4-b]pyrazine, TMS(1R,2S,8As)-8-oxo-1-carboxymethyl-1,2,5,5., 2-Nonynoic acid, Silanediol, dimethyl-Phenazine, 3-Heptanol, 2,4-dimethyl-Squalene (Yu et al., 2005) Hence, GC-MS analysis of endophytic fungal extracts reveals crucial bioactive compounds present in the endophytes that gives insights into their therapeutic applications.

Peak	R. Time	Area	Area%	Height	Height%	Name	Base m/z
1	2.519	210891	3.57	257435	10.56	2-Nonynoic acid	44.00
2	2.706	1254528	21.26	836737	34.33	Silanediol, dimethyl-	77.05
3	22.559	4043235	68.50	1108345	45.48	Phenazine	180.05
4	22.920	116266	1.97	68472	2.81	3-Heptanol, 2,4-dimethyl-	73.05
5	25.466	277319	4.70	166167	6.82	Squalene	69.10
		5902239	100.00	2437156	100.00		

Fig. Guilandina stem extract PDB crude)

Antimicrobial Activity

The antibacterial potential of L. irregularis extracts was evaluated against Staphylococcus sp. (1564), Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, and Klebsiella sp. 4151 using the disc diffusion method (Gond et al., 2012). Sterile discs were impregnated with extracts and cultured in Mueller–Hinton Agar (MHA) plate which were formerly seeded by test organisms. Distilled water disc served like negative control and Streptomycin disc served like positive control. These plates incubated in 37°C for one day, and measured the zone of inhibition. Minimum inhibitory concentration (MIC) assays were also performed using extracts of 125, 250, and 500 µg/ml concentrations.

Serial No.	Bacterial Strain	Control Stock	50% 500 ml	40% 250 ml	30% 125 ml
1	**Pseudomonas**	-	+	-	+
2	**E. coli**	-	+	-	+
3	**Klebsiella**	-	+	-	+
4	**Staphylococcus**	-	+	-	+

Fig. MIC values of crude extract of endophytic fungus L. irregularis

Serial No.	Bacterial Strain (Crude extract = 1.4 cm.)	Control Stock	50% 500 ml	40% 250 ml	30% 125 ml
1	**Pseudomonas ATCC 27853**	0.3 cm.	0.2 cm.	0.4 cm.	0.4 cm.
2	**E. coli ATCC 25922**	0.2 cm.	0.3 cm.	0.6 cm.	0.5 cm.
3	**Klebsiella 4151**	0.3 cm.	0.3 cm.	0.4 cm.	0.1 cm.
4	**Staphylococcus 1564**	0.6 cm.	0.4 cm.	0.3 cm.	0.5 cm.

Fig. Sensitivity of crude extract of endophytic fungus L. irregularis of G. bonduc

Antioxidant Properties:

It is commonly recognised that antioxidant chemicals can effectively prevent cellular damage caused by reactive nitrogen and oxygen species (RNOS). The DNA damage, cell degeneration, and cancer brought by ROS (Huang et al., 2007; Seifried et al., 2007). ROS-related illnesses like cancer, atherosclerosis, cardiovascular disease, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, etc., can be resolved by antioxidants. By the use of methanol as solvent, DPPH (2,2-Diphenyl-1-Picrylhydrazyl) technique was used to measure the antioxidant activity.

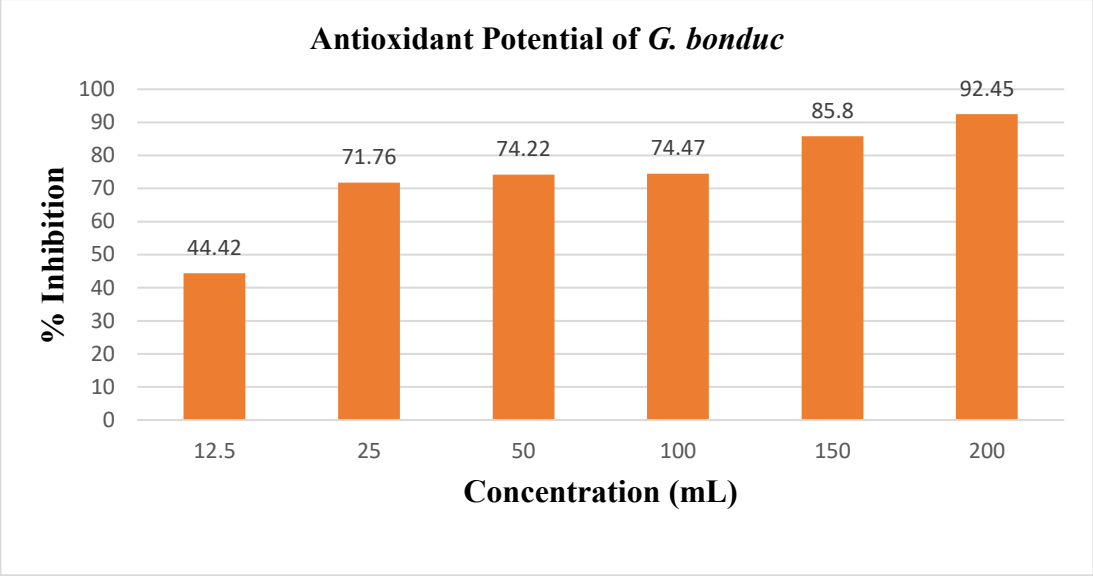

Fig. Antioxidant Potential graph of crude extract of L. irregularis from G. bonduc

Results and Discussion

In this study, the endophytic fungi Lasiodiplodia irregularis isolated from different plant parts of Guilandina bonduc are subjected for the extraction of the secondary metabolite in PDB media. The endophyte was identified by morphological as well as molecular methods using both universal and specific primers. Lasiodiplodia irregularis was assigned for Accession no. NFCCI – 6137. The culture of the endophytes is deposited in the Agharkar Research Institute (ARI) Pune. Endophytic fungi are well-known for the source of new bioactive chemicals and secondary metabolite according to Fox & Howlett, 2008. Various endophytic fungi have been found to produce new metabolites with biological beneficial compounds in the form of antibacterial, antifungal, antiviral, anti-inflammatory, and anticancerous properties that were derivative of alkaloids, steroids, flavonoids, and terpenoids. (B. Guo et el., 2008).

The fungal extract shows the presence of various chemical compounds and detected when applied thin layer chromatography (TLC) as a preliminary detection of the compounds present in the crude extract. This method depends on Retention factor (Rf) was the major parameter that can be extracted from a TLC investigation. The solution was used as mobile phase whereas Silica coated plate was the stationary phase in thin layer chromatography.

This measurement was done on the ratio of the distance travelled by the solvent front to the centre of a spot and it actually describes the migration of components over a TLC plate. These distances were measured from the beginning point. (J. Sherma, 2004). Spots represent chemical compounds present in the secondary metabolites of fungal extract. The chemical compounds have been extracted from the secondary metabolites of fungus was insoluble in water so the methanol was used as best solvent for the separation purpose of compounds. After that, the chemical compounds were detected by GC-MS chromatography. The GC-MS analysis shows various chemicals with retention times (RTs). It was occurred after the inoculation and incubation of Lasiodiplodia irregularis for at least 20 days in PDB media.

The variations between GC-MS and TLC through the use of a solid stationary phase, such as silica, and a liquid mobile phase, thin layer chromatography used to identification both organic and inorganic materials. GC-MS is utilized for the analysis and separation of volatile organic compounds and gases.

Both the gaseous mobile phase and the liquid stationary phase are necessary to work. Compounds in any mixtures can be successfully separated and identified using GC-MS, which is a powerful tool in modern analytical chemistry. (M. Gallo and P. Ferranti, 2016). Because the capillary column has a strong resolution, the GC-MS approach, which is used to identify low-molecular-weight molecules, is exact.
Although the TLC approach is less precise and has the drawback because of unable to separate the isomers. The TLC technique may be applied to screen for quick detection of food, drink, and vegetable extracts, while GC-MS is more expensive but more sensitive and yields significantly more information. Therefore, TLC is relatively the least expensive practical technique utilized in laboratories. (R. Oprean et el., 1989). Fungal endophytes contain a variety of secondary metabolite, in which some have beneficial properties according to Bills and Polishook, 1991. There are various studies based on use of GC-MS for the identification of chemicals produced by the fungal endophyte (RR Hateet, 2020).
Total twenty-five chemicals were reported by GCMS analysis of the Lasiodiplodia irregularis extract shown in table 4 and 6 (figure 5) in the form of : 2-Nonynoic acid, Silanediol, dimethyl- , Phenazine, 3-Heptanol, 2,4-dimethyl- , Squalene, 4- Vinylcyclohexene diepoxide (isomer 2), Silanediol, dimethyl- ,[R*,R*-(E)]-, Squalene,Khusimyl methyl ether, Solenophene-2-carboxylicacid, trimethylsilyl, 9,19-Cyclolanost-24-en-3-ol, acetate, (3.beta), (3S,6aR,6bR,8aS,12S,14bR)-4,4,6a,6b,8a,11,1H,2H,3H,4H-Pyridol[3,4-b]pyrazine, TMS(1R,2S,8As)-8-oxo-1-carboxymethyl-1,2,5,5., 2-Nonynoic acid, Silanediol, dimethyl-Phenazine, 3-Heptanol, 2,4-dimethyl-Squalene. Similar observation was also reported by Yu et al., 2005.
The fungal extracts of the tested species are found to contain the compound 2- Nonynoic acid. It is unsaturated, medium chain fatty acid and also known as non-2-enoic acid, nonylenic acid, or alpha nonynoic acid. One of the peculiar characteristics that it is also detected in Mushrooms, and primarily used as flavouring agent and preservative in the food industry, skincare products, as a surfactant insecticide formulation, in the synthesis of special polymers and certain pharmaceuticals. It is also utilized as a biochemical reagent in life science research and also have the potential for drug development due to its unsaturated fatty acid structure. (H. G. Rodrigues, 2010)
Silanediols are primarily used in adhesives, sealants, paints and coatings due to their stable, moisture-curing network formation. This metabolite is particularly applicable for CO_2 fixation into cyclic carbonates and activating nitroalkenes in conjugate addition reactions. Its also contains the potential as molecular recognition tools and explored for bio-inspired enzyme inhibition. This study have been showed that the identification of many chemical compounds in the tested fungal extract which were different from the compound recorded in crucial studies. Endophyte have major sources of novel and distinctive secondary metabolite, who shows itself as a wonderful source of medications with anti-inflammatory, antioxidant, anticancer, antibacterial, and antidiabetic properties. The creation of natural secondary metabolites from endophytic fungi may also help to protect the existence of natural resources that's why demanding is in hike of such products for being plant-based as well as thoroughly natural.

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