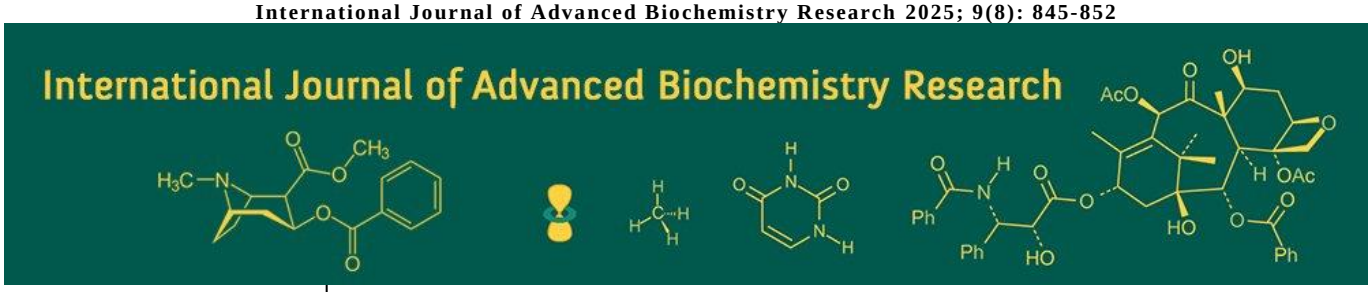

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Antibacterial properties of selected natural dyes against *Pseudomonas aeruginosa* and *Bacillus subtilis*

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Abstract
Natural dyes extracted from plant samples of *Beta vulgaris*, *Curcuma longa*, *Terminalia arjuna*, *Delonix regia*, *Bixa Orellana*, *Morus indica*, and fungus *Penicillium pupurogenum* were evaluated against the clinical pathogens of *Pseudomonas aeruginosa* and *Bacillus subtilis.* The plant samples were collected from in and around Coimbatore region. The fungal culture was obtained from Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. Clinical pathogens of bacteria were obtained from the PSG Medical College, Coimbatore, Tamil Nadu, India. Agar well diffusion method has been used to determine the antimicrobial activities. The study revealed that five percent methanolic concentration of *T. arjuna* bark extracts was found to be highly effective against *P. aeruginosa* with inhibition zone of 14.01 mm, and *B. subtilis* with 14.50 mm compared to other dye extracts. Streptomycin (30 µg/ml) exhibited zone of inhibition of 16.50 mm. The degree of inhibition varied with the concentrations of compounds extracted from dye powder on the clinical test organisms.

Keywords: Antimicrobial effect, natural dyes, plant extracts, gram positive, gram negative

Introduction
Textile processing remains one of the most significant industrial activities worldwide, spanning both developed and developing economies. However, the textile sector is regarded as one of the most complex and resource-intensive manufacturing industries (Ali *et al.*, 2021) [25]. The use of natural dyes for textile dyeing purposes decreased to a large extent after the discovery of synthetic dyes in 1856. At the start of the twentieth century, natural dyes were nearly abandoned due to a significant decrease in the cost of synthetic dyes (Holkar *et al.*, 2016) [24]. With an estimated 10-20 million tonnes used in industry, synthetic dyes now predominate. They pose major health risks and disturb ecological balance due to their production and use, which generates a lot of waste, including 15-20% of unfixed dyes released into wastewater (Sosa-Martínez *et al.*, 2020) [26]. Natural dyes, on the other hand, are typically more environmentally friendly and have superior biodegradability. They are easily obtainable, renewable, hypoallergenic, non-toxic, and non-carcinogenic (Joshi & Kuriyal, 2023) [27].

In addition to their eco-friendly and biodegradable qualities, plant-based natural dyes have recently become more cost-effective than synthetic ones (Sadeghi & Mozafarpour, 2024; Yadav, Kumar, & Singh, 2024) [29, 30]. However, issues with natural dyes include a lack of thorough scientific knowledge and standardised dyeing methods, duller hues, inconsistent results, and poor colour fastness (Gulrajani & Deepti, 1992; Samanta *et al.*, 2003) [5, 6].

Beyond plants, pigments of microbial origin have emerged as a promising sustainable alternative for dye production. Microbial pigments not only provide vivid coloration but also display antimicrobial, antioxidant, and anticancer properties, making them valuable for both industrial and biomedical applications (Venil *et al.*, 2020) [28]. Unlike synthetic colorants, microbial dyes are derived via environmentally friendly processes and can be produced at scale under controlled fermentation conditions. Recent studies highlight the potential of bacterial and fungal pigments for sustainable applications in textiles, food, leather, and pharmaceuticals (Duran *et al.*, 2021; Kucha *et al.*, 2022) [31, 32].

Materials and Methods

Maintenance of microbial cultures

The bacterial clinical pathogens utilized in this research were sourced from PSG Medical College in Coimbatore, Tamil Nadu, India. The pathogens tested in the study were preserved on their respective slants and kept refrigerated at 4 °C. Cultures were regularly transferred to fresh slants to maintain viability for ongoing experiments. Antimicrobial activity of pigments were examined using the agar well diffusion bioassay method against *Pseudomonas aeruginosa*, a Gram negative bacterium; *Bacillus subtilis*, a Gram positive bacterium were selected due to its popularity as a test organism and its resistance to common antimicrobial agents.

Preparation of plant extracts

Six plants and one fungal species were selected for dye extraction. The plant materials were collected from the Coimbatore region and the fungal culture was sourced from the Department of Agricultural Microbiology at Tamil Nadu Agricultural University, Coimbatore. The dried and powdered plant samples were extracted overnight at room temperature using 80% methanol, a polar solvent. The extracts were passed through Whatman No. 1 filter paper for filtration and subsequently concentrated under vacuum using the rotary evaporator. The dried extracts were stored in tightly sealed bottles refrigerated until antimicrobial testing. Similar extraction methods and sources have been applied in recent studies highlighting plant and fungal dye extraction and their applications (e.g., fungal pigment extraction and plant pigment extraction methods) (Chanda *et al.*, 2011) [13].

Preparation of test samples

Two different concentrations *viz*., 2 and 5 percent (20 mg/mL and 50 mg/mL) dye extracts were used for the antibacterial assays of clinical pathogens. Fifty gram of powdered plant parts soaked in 100 ml of methanol overnight.

The extracts were passed through Whatman No. 1 filter paper for filtration, solvent evaporated by vaccum evaporator and the residue obtained was dissolved using distilled water and concentrations of 2 and 5 percent prepared and stored for further works (Shrififar *et al.*, 2003) [15].

Preparation of test controls

Sterile distilled water without any test compound served as the negative control, while streptomycin at a concentration of 30 µg/ml was used as the positive control.

Preparation of culture media

The bacteria were cultured and maintained on respective medium.

For the bioassay, a loopful of the bacterial cell was inoculated into 100 ml of the respective broth. The conical flasks were incubated at a temperature of 37 °C for 24 h for bacteria.

Agar well diffusion assay

The sterilized nutrient medium was poured into petri dishes and left to solidify. The pour plate technique was applied to two distinct bacteria: *Pseudomonas aeruginosa*, a Gram-negative species, and *Bacillus subtilis*, a Gram-positive species. Each petri dish was then sectioned into three equal parts using a marker. Wells with a diameter of 6 mm were carefully created in each section using a sterile cork borer. For each bacterial strain, 20 µl of the prepared plant extract was added into the wells using a sterile dropper pipette. Each treatment was repeated three times for accuracy. The plates were incubated at appropriate conditions for 24 hours, after which observations were recorded. The antimicrobial effect was assessed by measuring the clear zone of inhibition surrounding the wells, indicating an area where bacterial growth was prevented. The diameter of these inhibition zones (DIZ) was measured, and the average DIZ was calculated as described by Iqbal *et al.* (1998) [16].

Results and Discussion

Table 1 shows the antibacterial properties of *B. vulgaris*, *C. longa*, *T. arjuna*, *D. regia*, *B. orellana*, *M. indica* against selected two different bacteria, *Pseudomonas aeruginosa* and *Bacillus subtilis*. The degree of inhibition varied with the concentrations of compounds extracted from dye powder on the two pathogenic bacteria.

Five percent concentration of *T. arjuna* bark extracts was found to be highly effective against *P. aeruginosa* at the inhibition zone of 14.01 mm diameter compared to other plant extracts. This was followed by *C. longa* which recorded the zone of inhibition of 13.02 mm, The extracts of *M. indica*, *B. orellana*, *B. vulgaris*, *D. regia* exhibited the inhibition zone of 13.01 mm, 9.00 mm, 4.00 mm and 2.00 mm respectively. Streptomycin (30 µg/ml) exhibited zone of inhibition of 16.50 mm (Fig. 1). Two percent concentration of *T. arjuna* showed a zone of inhibition of 7.00 mm compared to other plant extracts at the same concentration.

The extracts of *C. longa*, *M. indica*, *B. orellana*, *B. vulgaris*, *D. regia* produced a zone of inhibition of 6.00 mm, 6.00 mm, 3.00 mm, 1.00 mm and 1.00 mm respectively.

Compounds extracted from *T. arjuna* was remarkably effective at five percent concentration against *B. subtilis* with the inhibition zone of 14.50 mm compared to other dye extracts. *M. indica* and *C. longa* imparted the inhibition zone of 12.01 mm diameter. This was followed by *B. orellana B. vulgaris D. regia* which exhibited the inhibition zone of

5.00 mm, 2.00 mm and 1.00 mm respectively (Fig. 2). Two percent concentration of *T. arjuna* showed the diameter of 9.00 mm, followed by *M. indica*, *C. longa*, *B. vulgaris*, *B. orellana* which showed the inhibition zone of 7.00, 5.00 mm, 1.50 mm, 1.00 mm respectively. Streptomycin (30 µg/ml) exhibited zone of inhibition of 16.00 mm.

According to Hung and Chung (2003), presence of tannins in the fruit extract of *T. bellerica* might have prevented the development of microorganisms by precipitating the microbial protein and making nutritional proteins unavailable for them. Hagerman and Butler (1981) claim that tannins can suppress cellular protein synthesis by forming stable, irreversible complexes with proline-rich proteins. The existence of particular functional groups linked to Terminalia arjuna's high therapeutic value may be the cause of its therapeutic potential.

Naqvi *et al.* (2010) observed that the crude extract of *Terminalia chebula* exhibited an 82.35% inhibition rate against *Staphylococcus aureus*. Additionally, its aqueous, ethyl acetate, and n-butanol fractions produced inhibition zones ranging from 5-10 mm and 16-20 mm, respectively.

Antibacterial activity was also noted against other pathogens, including *S. aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Bacillus* species.

The most striking feature of the present findings is that apart from using synthetic dyes, natural dye extracts have a good scope because of the antimicrobial properties of plant extracts.

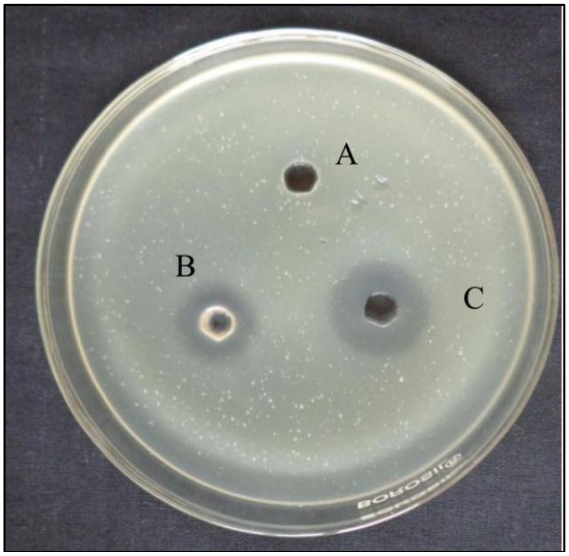


Fig 1: Zone of inhibition of *Terminalia arjuna* (5%) against *Pseudomonas aeruginosa* in nutrient agar. (A) control; (B) *T. arjuna;* (C) streptomycin

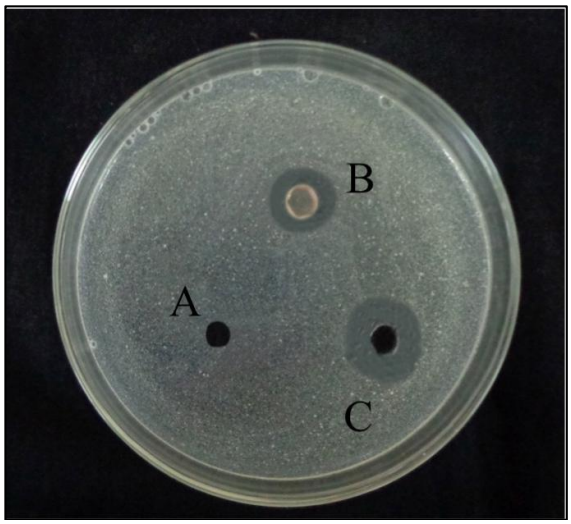
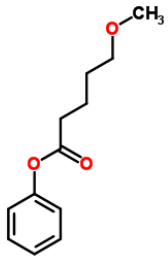
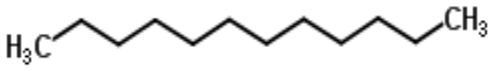
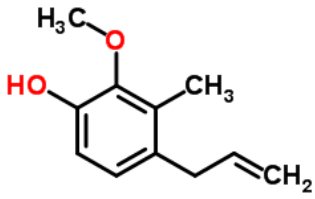
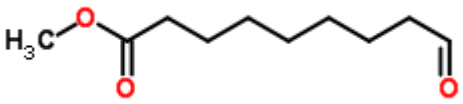
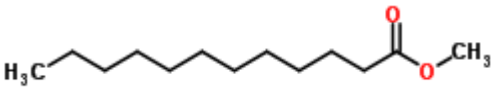
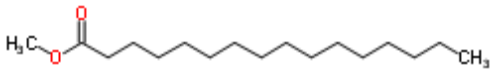
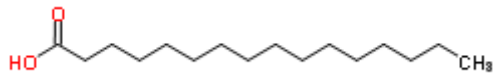
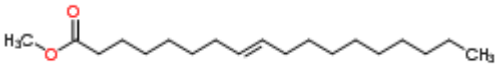
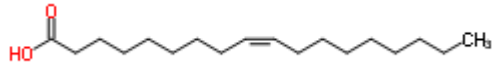
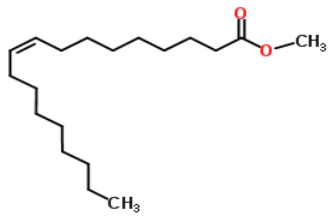


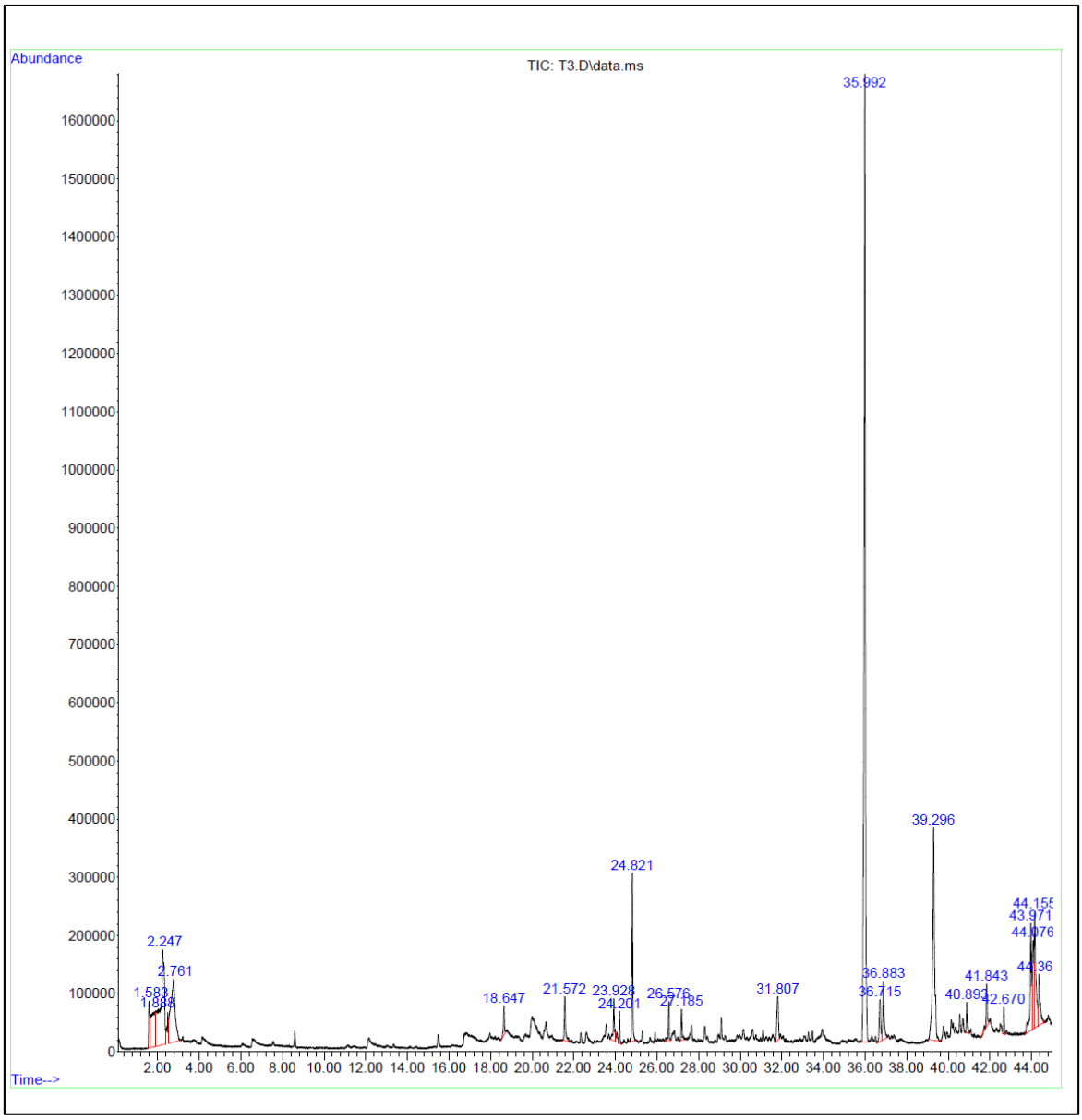
Fig 2: Zone of inhibition of *T. arjuna* (5%) against *B. subtilis* in nutrient agar. (A) control; (B) *T. arjuna;* (C) streptomycin

Table 1: Antibacterial activity of tested natural dyes

Sl. No.	Natural dye extracts	Zone of inhibition (mm)			
		P. aeruginosa		*B. subtilis*	
		2%	5%	2%	5%
T1	*B. vulgaris*	1.00^{e} (1.22)	4.00^{f} (4.95)	1.50^{e} (1.42)	2.00^{f} (3.70)
T2	*C. longa*	6.00^{c} (2.54)	13.02^{c} (8.58)	5.00^{d} (2.34)	12.01^{d} (8.24)
T3	*T. arjuna*	7.00^{b} (8.22)	14.01^{b} (8.88)	9.00^{b} (3.08)	14.50^{b} (9.04)
T4	*D. regia*	1.00^{e} (1.22)	2.00^{g} (3.69)	0.00^{g} (0.71)	1.00^{g} (2.85)
T5	*B. Orellana*	3.00^{d} (1.87)	9.00^{e} (7.18)	1.00^{f} (1.22)	5.00^{e} (5.45)
T6	*M. indica*	6.00^{c} (2.54)	13.01^{d} (7.92)	7.00^{c} (2.74)	13.01^{c} (8.56)
T7	*P. pupurogenum*	0.00^{f} (0.71)	0.00^{h} (1.63)	0.00^{g} (0.71)	0.00^{h} (1.66)
T8	Streptomycin	16.50^{a} (4.11)	16.50^{a} (4.11)	16.00^{a} (9.48)	16.00^{a} (9.48)
	SE (d)	0.03	0.04	0.03	0.03
	CD (0.05)	0.06	0.08	0.05	0.07

Table 2: GC-MS identified components of the *T. arjuna* bark dye extract (Compounds are listed in ascending order of Retention Time)

Peak No.	Compound	RT (min)	Molecular formula	Molecular structure **
1	Pentanoic acid, 5-methoxy-, phenyl ester **Synonym :** Phenyl 5-methoxypentanoate	2.247	$C_{12}H_{16}$	
2	Dodecane	18.647	$C_{12}H_{26}$	
3	Methyleugenol **Synonym :** 4-allyl-2-methoxy-3-methylphenol	23.928	$C_{11}H_{14}$	
4	Nonanoic acid, 9-oxo-, methyl ester **Synonym :** Methyl 9-oxononanoate	24.821	$C_{10}H_{18}$	
5	Dodecanoic acid, methyl ester **Synonym :** Methyl laurate	27.185	$C_{13}H_{26}$	
6	Hexadecanoic acid, methyl ester **Synonym :** Methyl palmitate	35.992	$C_{17}H_{34}$	
7	n-hexadecanoic acid **Synonym :** Palmitic acid	36.883	$C_{16}H_{32}$	
8	8-octadecenoic acid, methyl ester **Synonym :** Methyl (8E)-8-octadecenoate	39.296	$C_{19}H_{36}$	
9	Oleic Acid	41.843	$C_{18}H_{34}$	
10	9-octadecenoic acid, methyl ester, (E)-	39.296	$C_{19}H_{36}$	



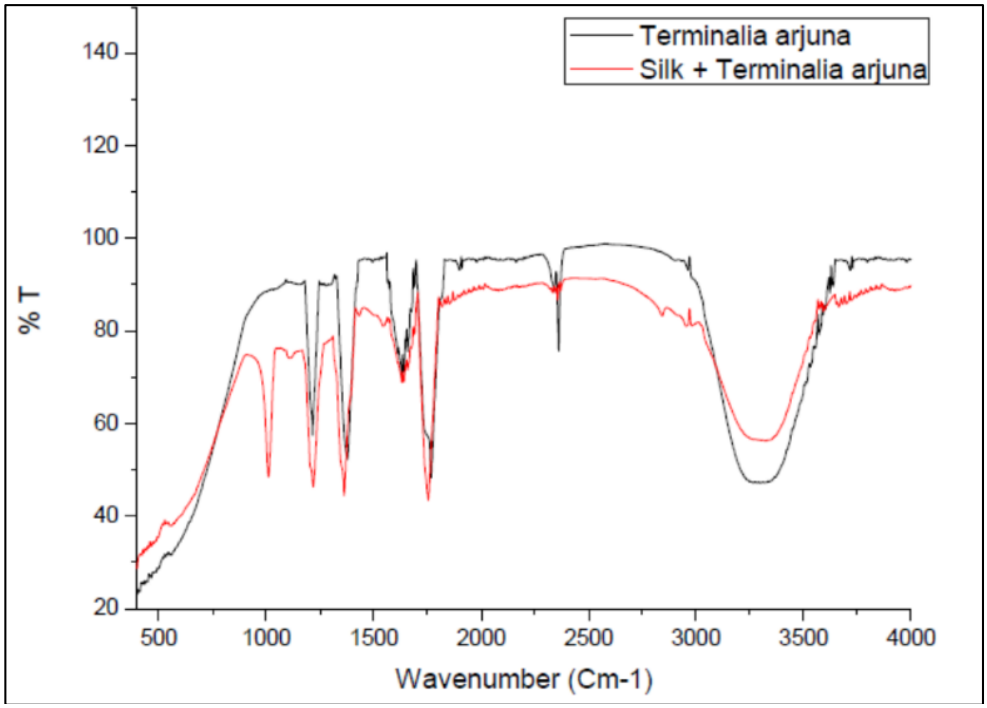


Fig 3: FTIR Spectra of *Terminalia arjuna*

Table 3: FTIR spectra of arjun tree, *T. arjuna*

Sl. No.	Frequency (cm^{-1})	Bond	Functional group
1	1162.87	C-H wag (-CH_2X)	Alkyl halides
2	1240.71	C-N stretching	Amine
3	1279.54	C-O stretch	Alcohols, carboxylic acids, esters, ethers.
4	1404.89	C-C stretch (in ring)	Aromatics
5	1633.41	N-H bend	1° amines
6	1758.10	C-H bending	Aromatic compound
7	2961.16	O-H stretching	Carboxylic acid
8	3342.03	O-H stretch	Phenol

Table 4: FTIR spectra of arjun tree, *T. arjuna* dyed mulberry silk

Sl. No.	Frequency (cm^{-1})	Bond	Functional group
1	1160.52	C-H wag (-CH_2X)	Alkyl halides
2	1249.05	C-N stretching	Amine
3	1263.15	C-O stretch	Alcohols, carboxylic acids, esters, ethers.
4	1408.92	C-C stretch (in ring)	Aromatics
5	1636.30	N-H bend	1°amines
6	1751.01	C-H bending	Aromatic compound
7	2955.38	O-H stretching	Carboxylic acid
8	3334.13	O-H stretch	Phenol

Gas chromatography-mass spectrometry (GC-MS) analysis was employed to characterize the bioactive antimicrobial constituents of *Terminalia arjuna*. Compound identification was achieved by calculating retention indices relative to a homologous series of n-alkanes and by interpreting mass spectral fragmentation patterns through comparison with reference spectral databases.

T. arjuna is an important medicinal plant widely used in ayurvedic formulation to cure coronary artery disease (CAD) and hypertension (Karthikeyan, *et al.*, 2003 and Patil *et al.*, 2011) [17, 18]. "Various parts of *T. arjuna*—especially the bark, fruit, leaves, seeds, and roots—are incorporated into traditional preparations such as decoctions and tonic mixtures for human consumption, often blended with water or milk and other beverages to promote overall health. Chemical analysis identified the wide range of bioactive compounds throughout the whole plant, including tannins, saponins, esters, sugars, steroids, various acids, and essential minerals. Both laboratory and clinical studies showed the plant's health promoting properties, especially its gastroprotective and anti-mutagenic potential (Devi *et al.*, 2007) [20]. *Terminalia arjuna* is particularly noted for its capacity to produce large quantities of wide range of secondary metabolites, such as phytosterols, lactones, flavonoids, phenolic compounds, tannins, and glycosides (Mandal *et al.*, 2013) [21].

In the present investigation, 23 components of *T. arjuna* bark extracts were found at the retention time of 1.583-44.364, the major compounds numbering ten having antimicrobial properties identified were pentanoic acid, 5-methoxy-, phenyl ester; dodecane; Methyleugenol; nonanoic acid, 9-oxo-, methyl ester; dodecanoic acid, methyl ester; hexadecanoic acid, methyl ester; n-hexadecanoic acid; 8-octadecenoic acid, methyl ester; 9-octadecenoic acid (Z)-, methyl ester and oleic acid with the retention time of 2.247, 18.647, 23.928, 24.821, 27.185, 35.992, 36.883, 39.296, 39.296 and 41.843 respectively (Table 2).

In this study, palmitic acid (also called as n-hexadecanoic acid) was found, and identified that it having antibacterial and cholesterol-lowering effects (Kiuchi *et al.*, 1984; Lowell, 1984) [22]. It has also shown strong activity against cancer cells like MCF-7, WRL-68, CaCo2, and Colo-320 DM, and can help protect the liver from damage caused by galactosamine.

Russel (1991) said that there are various fatty acids recognized to possess antibacterial and antifungal properties. Fatty acids dodecanoic acid, hexadecanoic acid and oleic acid have been characterized for their ability to combat bacterial and fungal infections. Also, unsaturated alcohols have been characterized for strong antibacterial (McGraw *et al.*, 2002), antifungal (Sheba *et al.*, 1999), antiviral (Sands *et al.*, 1979), and anti-inflammatory activity. *T. arjuna* showed that it contains less than one percent of triterpenes, 44 percent polyphenols, 12 percent sugars, 30 percent proteins.

Ramesh and Dhanaraj (2015) has reported that the compound 9-octadecenoic acid (Z), hexyl ester has various therapeutic properties. These include being an emollient, an anti-inflammatory, a hypocholesterolemic, a cancer-preventive, a hepatoprotective, nematicidal, insect-repellent, antihistaminic, anticoronary, and having antiarthritic effects. Additionally, many phenolic compounds are often used in medical treatments for issues like ingrown nails. They serve as neurolytic agents to relieve muscle spasms and chronic pain. They are also used in dermatological procedures such as chemical facial peeling.

The various range of bioactive compounds present in the bark provides strong proof for isolating individual phytochemical constituents. Conducting thorough biological activity assessments on these isolated compounds is expected to produce valuable insights and promising results, further validating the therapeutic potential of the bark extracts. Apart from using the toxic synthetic dyes, use of these natural dye extracts having antimicrobial properties might prevent human body from the attack of serious microorganisms and might reduce the probability of getting serious ailments/diseases, and might be those compounds could provide a better healthy life.

FTIR Spectra of natural dyes and dyed silk yarn

The result of FTIR spectrum of tested *T. arjuna* bark dye extracts and dyed silks recorded functional groups *viz.*, alkyl halides, amine, amide, alcohols, carboxylic acids, esters, ethers, aromatic groups, 1° amines, carboxylic acid, 2° amines and phenol groups at different peak area (Table 3 and 4).

The results are in line with those of Rathinamoorthy *et al.* (2011), who found that cotton textiles treated with extracts from Terminalia chebula had more carboxyl groups. This was explained by the extract's active ingredients, which included ascorbic acid and gallic acid. Carbonyl (C = O) stretching vibrations were present in the treated samples, as evidenced by absorption peaks in the 1600-1900 cm^{-1} range. Furthermore, the presence of ester groups was validated by absorption between 1760 and 1670 cm^{-1}. This implies that the hydroxyl (-OH) groups of cellulose and gallic acid interacted to form ester bonds in the treated fabric.

According to Nema *et.al.* (2012), FTIR spectrum of the *T. arjuna* leaves extract reported number of peaks lying between 3350.73 cm^{-1}, 2959.08 cm^{-1}, 2933.51 cm^{-1}, 2873.98 cm^{-1}, 1714.36 cm^{-1}, 1457.89 cm^{-1}, 1378.43 cm^{-1}, 1210.83 cm^{-1}, 1113.18 cm-1, 1070.84 cm^{-1}, 1041.56 cm^{-1}, 1028.36 cm^{-1} and 1009.84 cm^{-1} respectively.

Chaudhari and Mahajan (2015) used FTIR analysis to similarly identify different functional groups in the bark dyes of Terminalia arjuna. Alcohols, phenols, alkanes, carboxylic acids, primary amines, aromatic compounds, nitro compounds, and aliphatic amines were all confirmed by their investigation. The following specific absorption peaks were found: 3334.7 cm^{-1} for O-H stretching in phenols; 2931.3 cm^{-1} for C-H stretching in alkanes; 1724.06 cm^{-1} for C = O stretching in carboxylic acids; 1613.84 cm^{-1} for N-H bending in primary amines; 1520.98 cm^{-1} and 1445.39 cm^{-1} for C-C stretching in aromatic rings; 1351.86 cm^{-1} for N-O symmetric stretching in nitro compounds; and the range 1250-1020 cm^{-1} for C-N stretching in aliphatic amines. The expected functional groups of the corresponding compounds were well-aligned with these peaks.

The IR spectrum of *Terminalia arjuna* displays a strong absorption band at 3438 cm^{-1}, indicative of hydroxyl groups. The band observed at 2938 cm^{-1} corresponds to vibrations of saturated C-H bonds. Additional characteristic absorption peaks include those for carboxylic acids at 1716 cm^{-1}, amines at 1625 cm^{-1} and 1593 cm^{-1}, and phenols at 1257 cm^{-1} and 1171 cm^{-1}. Furthermore, the peak at 1051 cm^{-1} is attributed to glycosidic linkages (Krithiga *et al.*, 2014) [23].

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