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RESEARCH ARTICLE

Isolation, Characterization and Antimicrobial Activity of *Butea monosperma (Lam.)*

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ABSTRACT

The root and flowers of *Butea monosperma (Lam.)* were extracted with methanol. Extensive chromatographic separation and purification with the organic solvents was done. Four phytochemicals were separated and their structures were established based on various spectroscopic techniques. Isolated crude extract was subjected for antibacterial activity against gram-negative and gram-positive bacteria like *Bacillus megatarium, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* using standard protocols with MIC (Bacterial) in range of 7.5 to 25 µg/ml. The antifungal activity was also carried out against strains of *Alternaria*, *Fusarium* and *Aspergillus flavus* within the range between 10 µg/ml to 300 µg/ml MIC (Fungal). The results of antibacterial activity were compared with a standard antibiotic disc of ciprofloxacin and norfloxacin (5 to 30 µg/disc) against the same microbial strains. The results of antifungal activity were compared with nystatin (100 to 200 µg/disc) against the same microbial strains.

Keywords: *Butea monosperma, Flavonoids, Phenolic acid, Structure elucidation, Antimicrobial*

The study of medicinal plants opened the door to the development of purified and defined chemical compounds as dose-control players in the treatment of disease and in the understanding of disease mechanisms. Compounds, which emerged from the study of ethno-botanic extracts became important as medicines and were enabling pharmacologic tools in the elucidation of disease mechanisms [1]. The plant of *Butea monosperma (Lam.)* is very widely distributed in India and is used extensively in the folk medicine. The modern pharmacological study demonstrated that the plant has been used for many diseases. Methanol extract of *Butea monosperma (Lam.)* seeds, tested *in-vitro* showed significant anthelmintic activity [2], anticonvulsive [3], hepatoprotective [4], antiestrogenic potential [5], antifertility activity [6], anti-diarrhoeal activity [7] and antifungal activity [8]. Isolation and *in-vitro* antimicrobial efficiency of *Butea monosperma (Lam.)* seed oil is also observed [9-10]. "Pippali Rasayana", the mixture of Palash (*Butea monosperma (Lam.)*) and Pippali (Piper longum), is found useful in Giardiasis. *Butea monosperma (Lam.)* has been also evaluated for anti-stress activity and antifertility activity [11-15]. Most of pharmacological effects of *Butea monosperma* roots and flowers were attributed to its flavonoids and phenolic components. The chemical components of *Butea monosperma (Lam.)* have been widely investigated; flavonoids and triterpenoids were the main secondary metabolites. The effect of oral administration of the aqueous and alcoholic extracts of the leaves is assessed on stress, cognitive function and anxiety in albino rats [16-18]. The present paper deals with isolation and structural elucidation of three flavonoids and one phenolic acid. Four phytochemicals were isolated from the flowers and roots of *Butea monosperma (Lam.)*, their antimicrobial activities of extracted components were investigated, and results were compared with standards.

MATERIALS AND METHODS

General experimental procedures

Melting points were determined using the Scientific MP-D Melting point apparatus. IR spectra were determined on a Thermo Nicolet IR –200 spectrometer (ν_{max} in cm^{-1}). NMR spectra were recorded on a Bruker AMX 200 MHz instrument. ^{1}H NMR spectra were measured at 200 MHz and ^{13}C NMR spectra were measured at 200 MHz. The samples were prepared in deuterated DMSO and methanol. The chemical shift were given on δ (ppm) scale with tetramethylsilane as

an internal standard and coupling constant (J) were in Hz. The GC-EI-MS data were recorded on a Perkin Elmer Auto system XL GC+ with Elite - 5 (30 M x 0.25 mm x 0.50 µm) coloum. CC: Silica gel (S. D. fine chemicals limited India 60-120 mesh). Spots on TLC were visualized by UV (254 and 390 nm) and Potassium hydroxide as a spraying reagent, purchased from Molychem ltd. India.

Plant material

Roots and flowers of *Butea monospherma (Lam.)* were collected from campus of the Junagadh Agricultural University, India. An expert in the field of Botany authenticated the plant. *Butea monosperma (Lam.)* is native to tropical Southern Asia, India, Pakistan, Bangladesh, Nepal, Sri Lanka, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia and western Indonesia. It may be found throughout India up to a height of 1250 m, except in the arid zones.

Extraction and isolation

Roots and flowers of *Butea monospherma (Lam.)* were collected, shade dried at room temperature and ground in a manual mill. The powder was extracted with methanol using soxhlet-extracted for period of 24 hrs. The extract was filtered through a Buchner funnel with Whatman filter paper No.1. The filtrated was evaporate to dryness under reduce pressure using rotary evaporator. After evaporation of the solvent under reduced pressure, the residue was dissolved in methanol followed by column chromatography to preform purification of the component. A column of 1707 mm height and 15 mm inner diameter packed with neutral silica gel of mesh size 60-120 was used for the separation. The column was allowed to stabilize in ethyl acetate with overnight waiting period. Each collected fraction was analyzed by TLC method to ascertain the purification.

Antimicrobial Assay (disk diffusion method)

The extracted components were screened for their antimicrobial activity (antibacterial and antifungal screening) to evaluate the biological potentiality of extracted ingredients [19]. The methanol extract from *Butea monosperma (Lam.)* roots and flowers were tested against gram-positive and gram-negative bacteria like *Bacillus megatarium, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* and the antifungal activity was also carried out against strains of *Alternaria*, *Fusarium* and *Aspergillus flavus*. Various methods have been used to evaluate the antimicrobial activity from time to time [20]. The evaluation can be done by the methods like turbidometric method; agar streak dilution method; serial dilution method; agar diffusion method; agar cup method; agar ditch method and paper disc method. The Broth Dilution method was used to evaluate the antimicrobial activity. Agar cultures of the test microorganisms were prepared according to Mackeen *et al.* [21]. Samples were dissolved in methanol (1 ml). The test samples (10 µL) were loaded onto each Whatman filter paper disks (0.6 mm) and evenly placed on the agar surface previously inoculated with the suspensions of microorganisms to be tested. Standard disk of streptomycin sulphate (10µg/disk) was used as the positive control and DMSO was used as the negative control. The plates were inverted and incubated for 24 hours at 37^0C. Clear inhibition zones around the disk indicate the presence of antimicrobial activity.

The Broth Dilution Method [22] was then utilized the positive results for the determination of Minimum Inhibition Concentration (MIC). This test was performed in a sterile 96-well micro titer plates. Each active ingredient was diluted obtaining 1000 µg/mL concentration, as a stock solution. Each methanolic stock samples (10 µL) was transferred to micro titer plate well in duplicate at row. In primary screening, 500µg/mL, 250µg/mL and 125 µg/mL concentrations of the active ingredient were taken. The active ingredients found in this primary screening were further tested in a second set of dilution against all microorganisms.

The fractions found active in primary screening were similarly diluted to obtain 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL and 1.56 µg/mL concentrations. The highest dilution showing at least 99% zone of inhibition is taken as MIC. The result of this was much affected by the inoculums size. The test mixture should contain 10^6 cells/mL (bacteria) or 10^8 spores/mL (fungi).

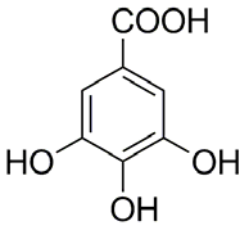
The MIC values were confirmed by the determination of Minimal Bactericidal Concentration (MBC) values according to method developed by Arias *et al* [23]. All wells in the MIC study, which did not show any growth of bacteria after incubation period were first diluted in fresh nutrient broth (1:4) and then sub-cultured onto the surface of freshly prepared nutrient agar plates (θ,15 mm). The plates were incubated for 24 hours at 37^0C. The MBC were recorded as the lowest concentration of the sample that did not permit any visible bacteria colony growth on the appropriate agar plate after the incubation period [24,25].

RESULTS AND DISCUSSION

Structure elucidation

Four phytochemicals have been isolated from the crude of *Butea monosoerma (Lam.)* Out of four phytochemicals 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one (quercetin); compound **2** was not reported earlier in the extracts of roots and flowers of *Butea monosoerma (Lam.)* under the specified experimental conditions, while compound 1, compound 3 and compound 4 were reported earlier in several work [26]. But their proposed structures were depicted here.

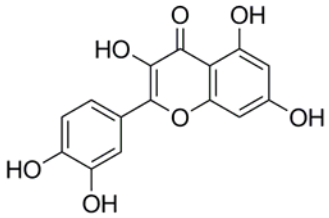
Compound **1** was isolated as white, yellowish-white, or pale fawn-colored crystals (methanol) with the molecular formula $C_7H_6O_5$, as determine by a molecular ion $M \rightarrow M^{+\bullet} + 2e^-$ at *m/z* 170.021523 generated from GC-EI-MS. The UV spectrum showed five



3,4,5-trihydroxybenzoic acid

Compound 1

Fig 1. Proposed structure of compound 1 based on spectral evidences



2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one

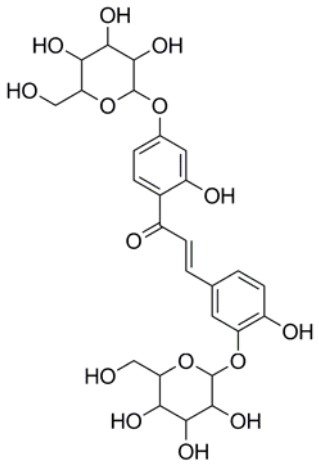
Compound 2

Fig 2. Proposed structure of compound 2 based on spectral evidences

characteristic bands at 212.1 nm, 267.5 nm, 353.2 nm, 367.3 nm and 380.5 nm. The FTIR spectrum displayed strong absorption bands at 2925.36 cm+, 2875 cm^{-1}, 3066.75 cm^{-1}, 1451.29 cm^{-1}, 1026.15 cm^{-1}, 701.62 cm^{-1}, 1703.08 cm^{-1}, 1245.44 cm^{-1}, 3287.22 $cm^{-1}$and 3372.78 cm^{-1} suggesting the presence of a flavonoid moiety. The ^{1}H NMR spectrum displayed signal at δ_H 12.24 (1H, s) with respect to TMS indicates the presence of two similar protons indicating presence of delocalization of electrons in the adjacent environment. This can be attributed to presence of –COOH group. A signal at δ_H 3.429 (1H, s) for the presence three protons of –OH group can be seen in the spectrum. Looking to the results of ^{1}H NMR, it is clear that the presence of aromatic ring with the three –OH and –COOH in the structure. A signal at δ_H 6.766 (1H, s) for the presence of aromatic hydrogen can also be seen. The proposed structure of compound 1 is shown in Fig 1.

Compound 2 was also isolated as white, yellowish-white crystals with molecular formula $C_{27}H_{32}O_{15}$, as determine by a molecular ion $M \rightarrow M^{+\bullet} + 2e^-$ at *m/z* 596.17412 generated from GC-EI-MS. The UV spectrum showed absorption maximum at 255.7 nm and 368.5 nm. The FTIR spectrum displayed strong absorption bands at 2936.97 cm^{-1}, 2859.73 cm^{-1}, 1492.40 cm^{-1}, 3040.24 cm^{-1}, 1580.72 cm^{-1}, 1014.83 cm^{-1}, 701.74 cm^{-1}, 1195.39 cm^{-1}, 1734.16 cm^{-1} and 3413.88 cm^{-1} suggesting the presence of a flavonoid moiety. In the ^{1}H NMR spectrum, signal at δ_H 3.108-3.607 (1H, m) relative number of 1H clearly indicates the presence of Ar –OH, Ar –H, and $–CH_2$ groups. Signal at δ_H 3.732-3.813 (1H, d, *J*=2) indicates presence of Ar –H. Two-protons' singlet for Ar-OH, appears at δ_H 5.465 (1H, s), two protons' doublet for Ar-H at δ_H 5.895 (1H, d, J=3). The spectrum also suggests that a three protons' multiplate for Ar-H, at δ_H 6.726 (1H, m) and one proton singlet indicates Ar-H at, δ_H 7.015 (1H, s), one proton singlet indicates Ar-H at, δ_H 7.187 (1H, s) and one proton doublet indicates –CH at, δ_H 7.545 (1H, d, J=9). ^{1}H NMR spectrum also showed two protons' doublet for Ar-H, -CH, which appeared at δ_H 8.064 (1H, d, J=0.8). The results also suggest the chain compound with an aromatic skeleton occupied with –OH and two ketonic oxygen in the structure. The proposed structure of compound 2 is shown in Fig 2.

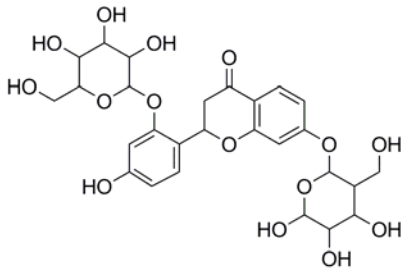
Compound 3 was isolated as white, yellowish-white crystals with the molecular formula $C_{15}H_{10}O_7$, as determine by a molecular ion $M \rightarrow M^{+\bullet} + 2e^-$ at *m/z* 302.236 generated from GC-EI-MS [19-20]. The UV spectrum showed absorption maximum at 272.3 nm and 373.3 nm. The FTIR spectrum displayed strong absorption bands at 2925.78 cm^{-1}, 2855.13 cm^{-1}, 3054.96



(*E*)-3-(4-hydroxy-3-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)phenyl)-1-(2-hydroxy-4-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)phenyl)prop-2-en-1-one

Compound 3

Fig 3. Proposed structure of compound 3 based on spectral evidences



2-(4-hydroxy-2-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)phenyl)-7-(4,5,6-trihydroxy-3-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)chroman-4-one

Compound 4

Fig.4. Proposed structure of compound 4 based on spectral evidences

cm^{-1}, 1459.60 cm^{-1}, 1015.03 cm^{-1}, 1195.39 cm^{-1}, 1734.16 cm^{-1} and 3413.88 cm^{-1} suggesting the presence of a flavonoid acid moiety. The ^1H NMR spectrums showed that a four protons' singlet for Ar-OH appeared at δ_H 4.958 (1H, s). One proton singlet for Ar-H appeared at δ_H 6.77 (1H, s) and one proton singlet for Ar-H appeared at δ_H 6.884 (1H, s). One proton singlet Ar-H for appeared at δ_H 6.891 (1H, s), one proton singlet for Ar-H appears at δ_H 6.923 (1H, d, J=1.4), one proton singlet for Ar-H appears at δ_H 7.613 (1H, d, J=4) and one proton singlet for Ar-OH appears at δ_H 12.45 (1H, s). ^1H-NMR spectrum of compound 3 is of suggestive that total seven types of 10 protons are present in the aromatic skeleton with presence of –OH and >C=O groups, because δppm for –OH showed correction due to the presence of delocalized л-electron in adjacent environment. The proposed structure of compound 3 is shown in Fig 3.

Compound 4 was isolated as white, yellowish-white crystals with the molecular formula $C_{27}H_{32}O_{15,}$ as determine by a molecular ion $M \rightarrow M^{+\bullet} + 2e^-$ at *m/z* 596.533980 generated from GC-EI-MS. The UV spectrum showed absorption maximum at 293.2 nm and 373.3 nm. The FTIR spectrum displayed strong absorption bands at 2923.15 cm^{-1}, 2853.66 cm^{-1}, 1470.73 cm^{-1}, 3069.94 cm^{-1}, 1524.22 cm^{-1}, 1108.32 cm^{-1}, 718.27 cm^{-1}, 1202.95 cm^{-1}, 1674.92 cm^{-1} and 3448.68 cm^{-1} suggesting the presence of a phenolic acid moiety. ^1H NMR spectrum suggest that a one proton multiplate for Ar-H appeared at δ_H 3.146 (1H, m), 16 proton multiplate for Ar-H,-CH_2 and Ar-OH appeared at δ_H 3.258-3.791 (1H, m). Two proton multiplate for Ar-H appeared at δ_H 3.863 (1H, m), one proton singlet for Ar-H appears at δ_H 5.305 (1H, s) and one proton multiplate for Ar-H appeared at δ_H 5.584 (1H, m). Two proton doublet for Ar-H appeared at δ_H 5.775 (1H, d, J=21), two proton multiplate for Ar-H appeared at δ_H 6.705 (1H, m) and two proton multiplate for Ar-H appears at δ_H 6.813 (1H, m). One proton singlet for Ar-H appears at δ_H 6.975 (1H, s) and one proton doublet for Ar-H appeared at δ_H 7.755 δ (1H, d, J=1). ^1H-NMR spectrum, suggest the presence of total 29 protons with 10 different type of proton, looking to the adjacent environment. The complex aromatic skeleton is present in the structure with –OH group shows δ_H value around 3.258-3.481 with respect to TMS. The proposed structure of compound 4 is shown in Fig 4.

Antimicrobial activity

Two fractions of roots and flowers of *Butea monosperma (Lam.)* viz. CVP-1 and CVP-2 were used for gram-negative and gram-positive bacteria like *Bacillus* megatarium, *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* and similarly both fractions were also used for fungal strains of *Alternaria*, *Fusarium* and *Aspergillus flavus* to evaluate the zone of inhibition. The results were compared with standard streptomycin sulphate disc. Zone of inhibition produced by two extracts were between 10 mm to 21 mm. Fraction CVP-1 was found significant against *Bacillus megatarium* (10 mm), *Bacillus cereus* (11 mm), *Bacillus subtilis* (11 mm), *Alternaria* (15 mm), *Fusarium* (18 mm) and *Aspergillus flavus* (10 mm). Fraction CVP-1 was therefore, found excellent against fungi strain *Fusarium* with 18 mm zone of inhibition. This could be attributed to the fact that fraction CVP-1 was obtained from flowers, which contains less concentration of phytochemicals. Fraction CVP-2 was found significant against *Bacillus megatarium* (13 mm), *Bacillus cerus* (20 mm), *Bacillus subtilis* (12 mm),

Table 1. Primary zone of inhibition study results of *Butea monosperma (Lam.)*

No.	Plant name/ Part used	Concentration 1 %					
		A	B	C	D	E	F
1	CVP-1	10 mm	11 mm	11 mm	15 mm	18 mm	10 mm
2	CVP-2	13 mm	20 mm	12 mm	21 mm	20 mm	11 mm

A: *Bacillus megatarium*, B: *Bacillus cereus* , C: *Bacillus subtilis*, D: *Alternaria*, E: *Fusarium*, F: *Aspergillus flavus*

Table 2. Antibacterial activity of *Butea monosperma (Lam.)*

No.	Plant name/ Part used	Minimal Bactericidal Concentration (MBC) (µg/mL)			
		A	B	C	D
1	CVP-1	7.5	6	3.5	15
2	CVP-2	10	6.4	7.5	25

A: Bacillus megatarium MTCC – 428, B: Bacillus subtilis MTCC – 441, C: Escherichia coli MTCC – 442, D: Pseudomonas aeuroginosa MTCC – 424

Table 3. Antifungal activity of *Butea monosperma (Lam.)*

No.	Plant name/ Part used	Minimal Fungicidal Concentration (MFC) (µg/mL)		
		A	B	C
1	CVP-1	10	150	50
2	CVP-2	50	180	300

A: *Alternaria* MTCC — 149, B: *Fusarium* MTCC — 2099, C: *Aspergillus flavus* MTCC — 277

Alternaria (21 mm), *Fusarium* (20 mm) and *Aspergillus flavus* (11 mm). Fraction CVP-2 was therefore, found excellent against fungi strains *Alternaria* and *Fusarium* with 21 mm and 20 mm zone of inhibition respectively. This could be attributed to fact that fraction CVP-2 was obtained from roots, which contain higher concentration of phytochemicals in comparison to flowers. The data of zone of inhibition were summarized in the Table 1. Antibacterial activity of fraction CVP-1 and CVP-2 were also carried out for two gram-negative bacteria *Bacillus megatarium* MTCC–428 and *Bacillus subtilis* MTCC–441 strains and against two gram-positive bacteria *Escherichia coli* MTCC–442 and *Pseudomonas aeruginosa* MTCC–424 strains. The results were compared with antibiotic disc of ciprofloxacin and norfloxacin with concentration of 5 to 30 µg/disc. It was clear from Table 2 that fraction CVP-1 was found excellent active against the bacterial strain *Escherichia coli* MTCC–442 with 3.5 µg/mL concentration. Similarly, fraction CVP-2 was found excellent active against the bacterial strain *Bacillus subtilis* MTCC–441 with 6.4 µg/mL concentration. Antifungal activity was also done using three fungi strains *Alternaria*, *Fusarium* and *Aspergillus flavus* and results were compared with standard of nystatin 100 to 200 µg/disc. Table 3 depicted here indicates that fraction CVP-1 was found excellent active against *Alternaria* MTCC—149 with 10 µg/mL concentration and fraction CVP-2 was found excellent active against *Alternaria* MTCC—149 with 50 µg/mL concentration.

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