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Quantification of Phytoconstituents and Isolation of Flavonoids from *Thespesia populnea* Bark Extracts

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Abstract

Flavonoids scavenged the free radicals and protect body from various diseases. The various types of flavonoids are present in the plants. More than 6000 flavonoids have been identified and still research requires to explore unidentified flavonoids. Hence, it was planned to to isolate and characterized the novel flavonoids present in bark of *Thespesia populnea*. In addition the quantification of phytoconstituents present in *Thespesia populnea* bark extract was also investigated. The petroleum ether, chloroform, acetone, ethanol and aqueous extract of barks were prepared. All the extracts were screened to quantify the total alkaloid, total polyphenol and total flavonol content. The ethanol extract was processed for column chromatography for isolation and characterization of flavonoids. The outcomes of total alkaloid exhibited present in chloroform, acetone and ethanol extract; while the total polyphenol and total flavonol were present in acetone, ethanol and aqueous extract. The ethanol extracts exhibited highest amount of polyphenol and flavonoids content compared to other extracts. The nine fractions (F1 to F9) were isolated from the ethanol extract of *Thespesia populnea*. The compound A and compound B were obtained after rechromatography. On the basis of various spectroscopic data, compound A and B isolated from *Thespesia populnea* extract were identified as 3,5,7-trihydroxy-4H-chromen-4-one and 2,2'-Trimethoxy-6,8-Dihydroxy-Isoflavone, respectively.

1 Introduction

Medicinal plants always play an important role **for** the development of health in mankind long before recorded history. Many people depend on the traditional medicine for their preliminary health care and treatment. Traditional medicine help us heal many ailments and almost always without any toxic side effects. Plants containing beneficial phytochemicals may supplement the needs of the human body by acting as natural antioxidants. Various studies have shown that many plants are rich source of antioxidants. For instance, vitamins A, C, E, and phenolic compounds such as flavonoids, tannins, and lignins, found in plants, all act as antioxidants[1, 2]. Flavonoids are one of the classes of heterocyclic natural compounds that are widely distributed in plant as glycosides or as free aglycones. It is a group of secondary metabolites derived from the phenylpropanoid pathway. Flavonoids are associated with a broad spectrum of health-promoting effects and are an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications. All these are due to its antioxidative, anti-inflammatory, immunomodulators, anti-mutagenic, hepatoprotective, wound healing, and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme functions. Presently, more than 6000 various flavonoids have been identified, and research in identification of new flavonoids from plants are interest area for researchers. The extraction of plant constituents is essential to isolate biologically active compounds and in understanding their role in disease prevention and treatment and in knowing their toxic effects as well.

Thespesia populnea, commonly known as the Indian tulip tree, is species of flowering plants in the mallow family, Malvaceae. It is distributed throughout coastal forests of India and also largely

grown as a roadside tree[3]. It is a shrub or medium-sized evergreen tree, up to 20 m tall with a dense crown. Bark is grey to brown, fissured, often knobby, and fibrous. It has heartshaped leaves glossy green in colour. Flowers are yellow with purple base, completely changing to purple when about to wither. Fruit a globose capsule. Seeds are pilose or powdery on the surface, flat, egg-shaped. It is currently place naturalized in tropical climates throughout the world. The tree well grows under full sunlight and tolerates drought conditions. The tree is valuable as coastal windbreak because it is it highly resistant to wind[4].

Different parts of *Thespesia populnea* are used in psoriasis, scabies, arthritis, rheumatism sprains, diarrhea, scabies, swellings, insect bites, hemorrhoids, warts, chronic dysentery, piles, cutaneous and anti-inflammatory diseases. Pulps of fresh fruits are applied for relief of migraine. The various chemical constituents were isolated from the *T. populnea* are Gossypol, 7 Hydroxy-2,3,5,6-tetrahydro-3,6,9-Trimethylnaphto [1,8-B,C] Pyran-4,8-Dione, Kaempferol, Quercetin, Kaempferol 3-glucoside, Quercetin 3-glucoside, rutin, Nonacosane, lupenone, myricyl alcohol, lupeol, β-sitosterol and β-sitosterol-β-D-glucoside, 5, 8-dihydroxy-7-methoxyflavone, 7-1ydroxyisoflavone and Thespone, Mansonones D E and F Populneol, Thespesin[5-8].

The present study was aimed to isolate and characterized the novel flavonoids present in bark of *Thespesia populnea*. In addition the quantification of phytoconstituents present in *Thespesia populnea* bark extract was investigated.

2 Materials and Methods

2.1 Plant material

The barks of *Thespesia populnea* was selected for our present work.

2.2 Collection and identification of plant material

The barks of *Thespesia populnea* were collected from the Village Bilantri & Sitamau Dist Mandsaur, M.P. India, medicinal garden of KNK College of Horticulture, Janta Colony, Rajaram Factory, Mandsaur, M.P. India. The plant was authenticated by Dr. S. N. Mishra (Sr. Scientist), K.N.K College of Horticulture. A voucher specimen of the plant was preserved in the herbarium for further reference.

2.3 Preparation of extracts

The powder of the bark of *Thespesia populnea* was packed in the Soxhlet apparatus and successively extracted with petroleum ether, chloroform, acetone, ethanol and distilled water until the completion of the extraction. The extract were filtered while hot, and the resultant extract was distilled in vacuum under reduced pressure in order to remove the solvent completely, and later dried in a desiccator. After that extracts of petroleum ether, chloroform, acetone, ethanol and aqueous were kept in air tight container for further study.

2.4 Quantitative analysis of phytoconstituents

2.4.1 Total alkaloid content

The petroleum ether, chloroform, acetone, ethanol and aqueous extracts (1mg/ml) was dissolved in 2N HCl and then filtered. 1 ml of this solution was transferred to separating funnel and washed with 10 ml chloroform (3 times). The pH of this solution was adjusted to neutral with 0.1 N NaOH. Then 5 ml of bromocresol green (BCG) solution and 5 ml of phosphate buffer were added to this solution. The mixture was shaken and complex extracted with 1, 2, 3 and 4 ml chloroform by vigorous shaking, the extract was then collected in a 10 ml volumetric flask and diluted with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. The whole experiment was conducted in three replicates.

Calibration curves of atropine

Accurately weighed 100 mg of atropine was dissolved in 100 ml of 2N HCl which gives the concentration of 1000 µg/ml. A set of standard solutions of atropine (1 to 10 µg/ml) were prepared. The different aliquots of Atropine standard solution was transferred to different separating funnels. Then 5 ml of pH 4.7 phosphate buffer and 5 ml of BCG solution was taken and the mixture was shaken with standard solution with 1, 2, 3, and 4 ml of chloroform. The standard solution were then collected in 10 ml volumetric flask and then diluted to adjust solution with chloroform. Now, the absorbance of the complex in chloroform was measured at 470 nm against the blank prepared as above but without atropine. Line of regression from atropine was used for estimation of unknown alkaloid content[9,10].

2.4.2 Total polyphenol content

Total polyphenol content was determined using colorimetric method. 1.0 ml of the prepared extract was oxidized using 2.5 ml of Folin- Ciocalteu reagent, and 2.0 ml of sodium carbonate solution (75 g/l) was then added to the reaction mixture. The absorbance readings were taken at 760 nm after incubation at room temperature for 2 h. The amount was calculated using the gallic acid calibration curve[11-13]. The results were expressed as gallic acid equivalent (GAE) mg per 100 ml of the sample (extract).

Calibration curves of gallic acid

Accurately weighed 100 mg of gallic acid was dissolved in 100 ml of distilled water which gives the concentration of 1000 µg/ml. 10 ml of this solution was taken and made up to 100 ml with gallic acid which contains the concentration of 100 µg/ml. Further 10 ml of this solution was taken and made up to 100 ml with gallic acid which contains the concentration of 10 µg/ml. 1 to 10 ml were taken from this solution and made up to 10 ml to get the concentration ranges of 1 to 10 µg/ml. Calibration curve

was plotted by mixing 1 ml aliquots of gallic acid solutions with 2.5 ml of Folin-Ciocalteu reagent and 2.0 ml of sodium carbonate solution (75g/l). The absorbance was measured after incubation at room temperature for 2 h at 760 nm using UV spectrophotometer, against blank solution.

2.4.3 Total flavonol content

Flavones and flavonols contents were analyzed by the colorimetric method. 9.8 ml of the prepared extract was mixed with a 10% solution of aluminum chloride (200 µl). After 30 min, absorption was measured at a 425 nm wavelength. The amount was calculated using quercetin calibration curve[11-13]. The results were expressed as the quercetin equivalent (QE) mg per 100 ml of the sample.

Calibration curves of quercetin

Accurately weighed 100 mg of quercetin was dissolved in 100 ml of distilled water which gives the concentration of 1000 µg/ml. 10 ml of this solution was taken and made up to 100 ml with quercetin which contains the concentration of 100 µg/ml. Further 10 ml of this solution was taken and made up to 100 ml with quercetin which contains the concentration of 10 µg/ml. 1 to 10 ml were taken from this solution and made up to 10 ml to get the concentration ranges of 1 to 10 µg/ml. Calibration curve was plotted by mixing 9.8 ml aliquots of quercetin solutions with a 10% solution of aluminum chloride (200 µl). The absorbance was measured 30 min at 425 nm using UV spectrophotometer, against blank solution.

2.5 Isolation of compounds from ethanol extract

The extract was packed to column chromatography applying silica gel (60-120 mesh size), & eluted with the following solvent ratios of Petroleum ether (PE): Ethyl acetate (EA), 100:0, 80:20, 60:40, 40:60, 20:80, 0:100, then with 100:0, 90:10, 80:20, 70:30, 60:40, 50;50, 40:60, 30:70, 20:80, 10:90, 0:100, EA: Ethanol (Eth). Then after it is eluted with 10:90 methanol (Mth): ethanol and finally with 100% methanol. The fractions (25 ml) were collected from the column. The elute collected were monitored by thin layer chromatography (eluent: EA-MeOH, 9:1 & 3:2) for homogeneity & the similar fraction were pooled together. The nine different fractions were collected & dried. The fraction F1, F2 & F3 were containing waxy material; the fractions F4, F5, F7 & F9 were powder but quantity was very little. The yield of fraction F6 and F8 were 380 mg & 260 mg, respectively. Further purification of selected fractions using rechromatographed. The F6 was eluted with the solvent EA-MeOH, 3:2 to yield compound A. The quantity of compound A was 110 mg. The F8 was eluted with the solvent EA-MeOH, 9:1 to yield compound B. The yield of compound B was 90 mg. The compound A and B were characterized by interpreting the data obtained from melting points, UV spectra, IR spectra, NMR spectra and Mass spectra[14,15].

3 Results and Discussions

3.1 Quantitative analysis of phytoconstituents

3.1.1 Total alkaloid content of Thespesia populnea

The petroleum ether, chloroform, acetone, ethanol and aqueous extracts of *Thespesia populnea* was evaluated for investigation of the total alkaloid content concentrations in extracts. Standard curve of atropine was calculated and plotted in distilled water for determining absorption data (Fig 1). From this Beer's law range and regression coefficient is determined. The linear equation of atropine was found to be y = 0.0138x (Fig 1).

The results of the total alkaloid content of the extracts examined are depicted in table 1. The total alkaloid content in extracts, expressed as atropine equivalents (AE). The total alkaloid content of chloroform, acetone and ethanol extract of *Thespesia populnea* were 22.41, 24.63 and 39.25 AE mg/gm, respectively. The alkaloid was absent in petroleum ether and aqueous extract. The ethanol extracts exhibited highest amount of total alkaloid content compared to other extracts. The outcomes of total alkaloid present in chloroform, acetone and ethanol extract of *Thespesia populnea* contribute the therapeutic property.

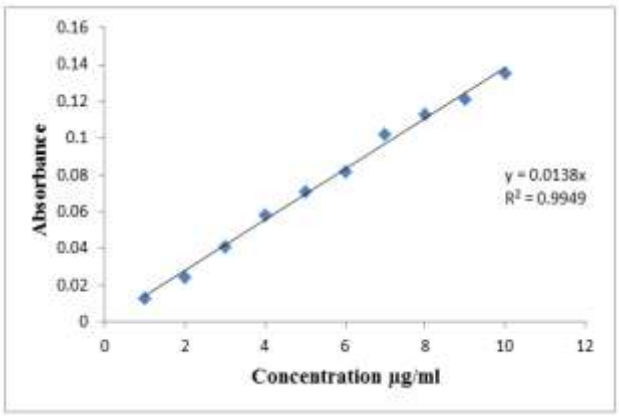


Fig 1: Calibration curve of atropine

Table 1: Determination of total alkaloid content of *Thespesia populnea*

Extract	Total alkaloid content (AE mg/gm)
Petroleum ether	--
Chloroform	22.41±0.15
Acetone	24.63±0.08
Alcohol	39.25±0.31
Aqueous	--

Data expressed as atropine equivalent (AE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

3.1.2 Total phenolic content of Thespesia populnea

The petroleum ether, chloroform, acetone, ethanol and aqueous extract of *Thespesia populnea* was evaluated for investigation

of the total phenolic content concentrations in extracts. Standard curve of gallic acid was calculated and plotted in distilled water for determining absorption data (Fig 2). From this Beer's law range and regression coefficient is determined. The linear equation of gallic acid was found to be y = 0.0395 x - 0.0052 (Fig 2). The results of the total phenolic content of the extracts examined, using Folin-Ciocalteu method, are depicted in table 2.

Table 2: Determination of total polyphenol content of *Thespesia populnea*

Extract	Total polyphenol content (GAE mg/gm)
Petroleum ether	--
Chloroform	--
Acetone	22.14±0.62
Alcohol	73.81±0.27
Aqueous	61.35±0.93

Data expressed as gallic acid equivalent (GAE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

The total phenolic content in extracts, expressed as gallic acid equivalents. The total phenolic content of acetone, ethanol and aqueous extract of *Thespesia populnea* were 22.14, 73.81 and 61.35 GAE mg/gm, respectively. The phenolic content in petroleum ether and chloroform extract was absent. The ethanol extracts exhibited highest amount of total polyphenol content compared to other extracts.

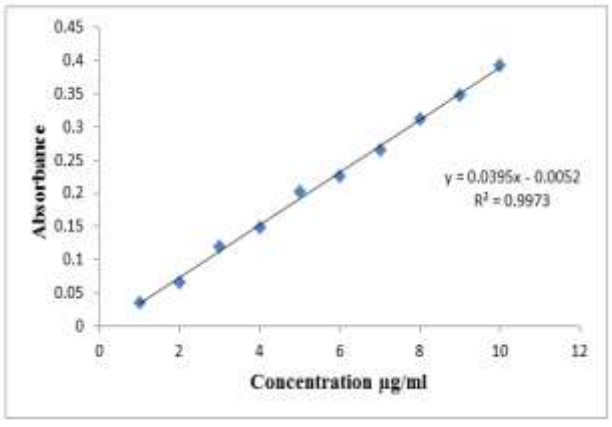


Fig 2: Calibration curve of gallic acid in distilled water

3.1.3 Total flavonol content of Thespesia populnea

The concentration of flavonoids in petroleum ether, chloroform, acetone, ethanol and aqueous extract of *Thespesia populnea* were determined spectrophotometrically using aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalents. Standard curve of quercetin was calculated and plotted in distilled water for determining absorption data (Fig 3).

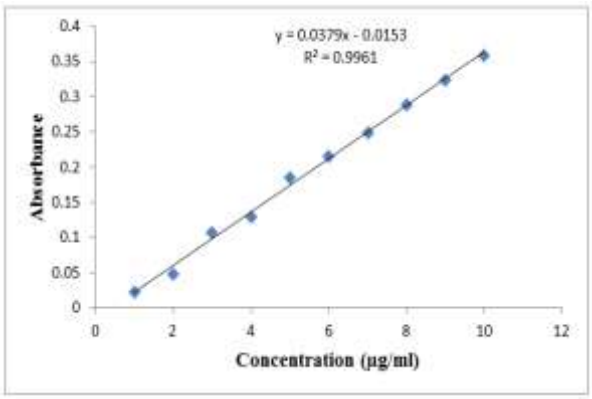


Fig 3: Calibration curve of quercetin in distilled water

From this Beer's law range and regression coefficient is determined. The linear equation of quercetin was found to be y = 0.0379x -0.0153 (Fig 3). The content of flavonoids identified in the tested extracts is shown in table 3. The concentrations of flavonoids in acetone, ethanol and aqueous extract of *Thespesia populnea* were 18.52, 59.12 and 42.36 QE mg/gm, respectively. The flavonol was absent in petroleum ether and chloroform extract. The ethanol extracts exhibited highest amount of flavonoids content compared to other extracts.

Table 3: Determination of total flavonol content of *Thespesia populnea*

Extract	Total flavonol content (QE mg/gm)
Petroleum ether	--
Chloroform	--
Acetone	18.52±0.32
Alcohol	59.12±0.09
Aqueous	42.63±0.21

Data expressed as quercetin equivalent (QE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

It is well documented that plant flavonoids and phenols in general, are greatly effective free radical scavenging and antioxidants. Polyphenol and flavonoids are used for the prevention of various diseases, which are mainly associated with free radicals. The phenolic compounds have been recognized as antioxidant and have been known to show medicinal activity as well as for exhibiting physiological functions[16,17].

It has been reported that compounds such as the flavonoids, which contain hydroxyl, are responsible for the radical scavenging effects of most plants. The mechanism of action of the flavonoids is through scavenging or chelating processes. It is well known that plant phenolics, in general are highly effective in free radicals scavenging, and they are antioxidants[18-20]. The therapeutic strategies targeting reactive oxygen species by antioxidants are being introduced into the treatment of different

diseases. The findings of total polyphenol and flavonol content of acetone, ethanol and aqueous extract of *Thespesia populnea* supports the study for the determination of its therapeutic property.

3.2 Characterization of Compound from extracts

3.2.1 Characterization of Compound - A

The melting point of isolated fraction was recorded to be >260 °C.

The sample solution was diluted with methanol. UV scan was done between 200 – 400 nm and the speed of instrument scanning was set as fast. The maximum absorbance λ_{max} obtained for the compound A at 272.5 nm

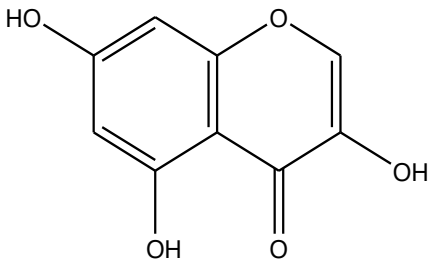
The IR spectrum of compound A showed absorption bands at 3309.85 (O-H, free hydroxyl group), 3197.98 (Cyclic C-H, str), 2989.66 (Cyclic C-H, str), 2850.79 (Ali- C-H, str), 2476.60 and 2198.85 (Alkyne group), 1708.93 (C=O, ester), 1639.49 (C-C ring stretch), 1280.73 (C-C stretching), 1068.56 (O-H, out of plane bend), 867.97 (monosubstituted in aromatic ring), 655.80 (out of plane ring C=C, bend).

The ^{1}H-NMR spectrum of compound A displayed the characteristic signals at δ_H 15.01 (O-H, 3, enol, s), 7.01 (H-2, s, ethylene), 5.94 (H, 6 & 8, s), 5.00 (OH-5 & 7, s).

The ^{13}C-NMR spectrum of compound A displayed the characteristic signals at 2-145.24 (C), 3-155.97 (C), 4-177.94 (C), 5-164.18 (C), 6&8-100.59 (C), 8a-160.84 (C), 4a-108.84 (C).

The mass data which showed m/z = 194.35 considered as 194 (100) [M^+] indicative of $C_9H_6O_5$.

Compound A was isolated and its molecular formula was determined as $C_9H_6O_5$ (m/z = 194 (100) [M^+]). The IR spectrum indicated the presence of hydroxyl (3419.16 cm^{-1}) and carbonyl functions (1708.93 cm^{-1}). The presence of the aromatic hydroxyl group and carbonyl were confirmed from the ^{13}C NMR spectra exhibited peaks at δ 155.97 to 164.18 and 177.94 respectively. The ^{1}H-NMR spectra exhibited peaks at δ 5.00, it suggested the presence of aromatic hydroxyl group. The ^{13}C NMR revealed higher δ 177.94, while absence of signal in ^{1}H-NMR inferred that carbon atom is free from proton. This statement confirmed the presence of carbonyl group at fourth position in ring. The ^{1}H-NMR exhibited peaks at δ 7.01 indicate the presence of benzene CH. It confirmed from the ^{13}C-NMR peaks from 145.24. The ^{1}H-NMR displayed the signal at δ 15.01 it demonstrated presence of enol at third carbon ring. The hypothesis can be clearly justified from the signal of ^{13}C NMR, it exhibited signal at 155.97. The structures was identified as flavonoids on the basis of extensive spectroscopic data analysis and by comparison of their spectral data with those reported in the literature. The compound characterized as 3,5,7-trihydroxy-4H-chromen-4-one (Fig 4).



3,5,7-trihydroxy-4H-chromen-4-one

Fig 4: Characterization of Compound - A isolated from *Thespesia populnea*

3.2.2 Characterization of Compound - B

The melting point of isolated fraction was recorded to be >320 °C.

The sample solution was diluted with methanol. UV scan was done between 200 – 400 nm and the speed of instrument scanning was set as fast. The maximum absorbance λ_{max} obtained for the compound A at 276.3 nm

The IR spectrum of compound B showed absorption bands at 3340.71 (O-H, free hydroxyl group), 2927.94 (Cyclic C-H, str), 2854.65 (Ali- C-H, str), 2364.73 (Alkyne group), 1732.08 (C=O, ester), 1369.46 (C-C ring stretch), 1284.59 (C-C stretching), 1010.70 (O-H, out of plane bend), 887.26 (monosubstituted in aromatic ring), 582.50 (out of plane ring C=C, bend).

The ^{1}H-NMR spectrum of compound B displayed the characteristic signals at δ_H 6.24 (H–7, s), 7.59 (H–6' s), 6.99 (H–5', s), 6.98 (H–4', s), 6.94 (H–3', s), 6.25 (H–7, s), 5.18 (O-H-6 & 8, s), 3.60 (CH_3–2 & 2', s).

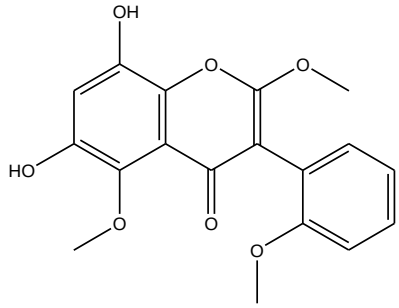
The ^{13}C-NMR spectrum of compound displayed the characteristic signals at 2-174.46 (C), 3-98.16 (C), 4-175.62 (C), 5-147.62 (C), 6-144.98 (C), 7-119.97 (C), 8-146.70 (C), 4a & 8a-115.57 (C), 1' & 6'-121.95 (C), 5'-125.69 (CH), 4'-102.96 (C), 3'-115.57 (CH), 2'-156.09 (CH), OCH_3-57.08, OCH_3-46.62.

The mass data which showed m/z = 344.26 (100) [M^+] indicative of $C_{18}H_{16}O_7$.

Compound B was isolated and its molecular formula was determined as $C_{18}H_{16}O_7$ (m/z = 344 (100) [M^+]). The IR spectrum indicated the presence of hydroxyl (3340.71 cm^{-1}) and carbonyl functions (1732.08 cm^{-1}). The presence of the aromatic hydroxyl group and carbonyl were confirmed from the ^{13}C NMR spectra exhibited peaks at δ 144.98 to 146.70 and 175.62, respectively. The ^{1}H-NMR spectra exhibited peaks at δ 5.18, it suggested the presence of aromatic hydroxyl group. The ^{13}C NMR revealed higher δ 175.62, while absence of signal in ^{1}H-NMR inferred that carbon atom is free from proton. This statement confirmed the presence of carbonyl group at fourth position in ring.

The ^{1}H-NMR exhibited peaks at ð 6.24, 7.59, 6.99, 6.98 and 6.95 indicate the presence of benzene CH. It confirmed from the ^{13}C-NMR peaks from 119.97 to 125.69. The ^{1}H-NMR displayed the signal at ð 3.60 it demonstrated presence of CH_3. The hypothesis can be clearly justified from the signal of ^{13}C NMR, it exhibited signal at 57.08 and 46.62. The structures was identified as flavonoids on the basis of extensive spectroscopic data analysis and by comparison of their spectral data with those reported in the literature. The compound characterized as 2,2'-Trimethoxy-6,8-Dihydroxy-Isoflavone (Fig 5).

The characterization results showed spectral data of possible flavonoids, and identified as 3,5,7-trihydroxy-4H-chromen-4-one and 2,2'-Trimethoxy-6,8-Dihydroxy-isoflavone.



2,2'-Trimethoxy-6,8-Dihydroxy-Isoflavone

Fig 5: Characterization of Compound - B isolated from *Thespesia populnea*

4 Conclusion

In conclusion, the observation and results obtained in present study indicates flavonoid fraction were namely 3,5,7-trihydroxy-4H-chromen-4-one and 2,2'-Trimethoxy-6,8-Dihydroxy-isoflavone present in *Thespesia populnea*. Hence the therapeutic properties of *Thespesia populnea* are probably due to presence of these flavonoids. In future more research is required to evaluate the pharmacological activity of these isolated compounds.

5 Conflict of interest

The authors declare that there are no conflicts of interest.

6 Author's contributions

MK performed the experimental work. DKJ carried out draft the manuscript.

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