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Pharmacognostical, Phytochemical Studies and *In vitro* antidiabetic Evaluation of Seed Extracts of *Casuarina equisetifolia* Linn.

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ABSTRACT

Casuarina equisetifolia is a fast growing, evergreen pine tree. It is a well-known xerophytic plant of the tropics and subtropics region. It is endemic to coastal area and its origin from Australia. The current study has been done to examine the Pharmacognostical, Phytochemical analysis and *In vivo* antidiabetic activity of successive solvent seed extract of *Casuarina equisetifolia*. The plant materials are collected from Coimbatore district and are authenticated by Siddha institute in Chennai. The pharmacognostical parameters such as Macroscopy, microscopy, powdermicroscopy, physicochemical constants and to determine the inorganic elements present in the seed. The seeds can be well dried and make into a coarse powder. Then it is subjected to continuous hot percolation method by using soxhlet apparatus. In successive solvent seed extraction, the extracts can be obtained from different types of polarity solvents namely non polar (hexane), mid polar (ethyl acetate) and polar solvent (ethanol). Its secondary metabolites are evaluated by preliminary Phytochemical analysis. The quantification of phyto constituents such as quercetin, Kaempferol, stigmasterol, Rutin, Ellagic acid are carried out by HPTLC, LC-MS. The antidiabetic activity of seed extract is confirmed by alpha amylase inhibitor assay and glucose uptake assay by using 3T3 L1 cell line.

Keywords: Glucose uptake assay, *Casuarinas equisetifolia*, 3T3 L1 cell line, Alpha amylase.

1. INTRODUCTION

Medicinal plants are also being considered for the treatment of diabetes. Many common drugs have been taken from prototypic molecules in medicinal plants. Metformin is an effective example of lowering oral sugar. Its development was based on the use of *Galega officinalis* in the treatment of diabetes. *Galega officinalis* is rich in guanidine, a hypoglycemic compound. Because guanidine is highly toxic for clinical use, alkyl Biguanides synthalin A and synthalin B were introduced as anti-diabetic agents in Europe in the 1920's but were discontinued after insulin became widely available. However, experience with guanidine and biguanides encouraged the development of metformin. To date, more than 400 conventional treatments for diabetic plants have been reported, although only a handful have received scientific tests and treatments for their effectiveness. The hypoglycemic effect of certain herbal supplements is confirmed in human models with animal type 2 diabetes. The World Health Organization (WHO) Committee on Diabetes has recommended that a study of alternative therapies be investigated. A major obstacle to integrating herbal medicine into modern medicine is the lack of scientific and clinical data proving its effectiveness and safety. There is a need to conduct clinical research on herbal drugs, to develop simple bioassays for environmental suspension, chemical and toxicity testing, and to develop a variety of animal toxicity and safety testing. It is also important to establish the active ingredient / components from this plant extraction.

India is the largest plant of *Casuarina equisetifolia* in the world and it is estimated that 500, 000 hectares are planted with *Casuarina* in the Andhra Pradesh, Orissa, and Tamil Nadu regions and the *Casuarinaceae* in India, which are *Casuarinales*, a distinct group of angiosperms [3].

Casuarina equisetifolia contains many active metabolites including carbohydrates, alkaloids, proteins, glycosides, saponins, phenolics, flavonoids, tannins, steroids, gum, reducing sugars and triterpenoids [4-5].

It was used for the treatment of constipation, cough, diabetes, diarrhea, gonorrhea, nervous disorders, rash, throat infections and stomach ulcers. However, the bark is used as an astringent and is also used for abdominal pain, diarrhea, rashes and nervous disorders. Leaf: was used as an antispasmodic for colic. Aerial components: were used as hypoglycemic. The seeds were used as Anthelmintic, Antispasmodic and Antidiabetic [6-8].

Therefore, current research has been conducted to evaluate the anti diabetic activity of *Casuarina equisetifolia* from seed extraction.

2. MATERIALS AND METHODS

2.1 Collection and Authentication

The freshly and mature seed was collected from the Coimbatore district at the date of 10.10.18. The sample was taxonomically identified and authenticated as Casuarinaceae, Code C06101801E Sidha Central research institute, Chennai.

2.2 Morphological Evaluation

Detailed investigation of morphological characters are its ensure the quality of crude drug. Investigation of morphological characters are refers to evaluation of plant material by means of size, shape, odour, taste, texture, fracture. The macroscopic characters of the sample were evaluated based standard text reference [9-100].

2.3 Microscopic Evaluation

Mature seeds are taken into 100 ml beaker its contain water then it boil for 2hours, transverse section carried out by free hand section method with help of sharp blade. the section are mounted in saffron dye and subjected into microscopic observations [11-12].

2.4 Powder Microscopy

Small amount of seed powder taken into microscopic slide. Then one drops freshly prepared 0.1% phloroglucinol added to this slide, followed by a drop of concentrated HCl and glycerol too after covered with cover slip finally its viewd under microscope. [12]

2.5 Physicochemical Analysis

In this investigation seed powder was subjected to the physicochemical studies there are Total ash, Acid insoluble ash, water soluble ash. The moisture content of raw drug is estimated by Loss on drying (105°C) method. Extractive value are measured by using various organic solvent. Foaming index are determine by followed by textual reference [13-15].

2.6. Preparation of Seed Extract

The seeds can be well dried and make into a coarse powder. 750gm coarse powder are subjected to continuous hot percolation method by using Soxhlet apparatus. In successive solvent seed extraction, by using different types of polarity solvents namely Hexane, ethyl acetate and ethanol. The extracts are filtered and concentrated by rotaory evaporator and dried [19].

2.7. Preliminary Phytochemical Analys

The following seed extracts are CHE, CEAE, CEET Subjected to preliminary Phytochemical screening for detection of the secondary metabolite and primary metabolite [20-22].

2.8. Quantification of Flavonoids and Sterols by HPTLC

HPTLC analysis of seed extract such as CEH, CEEA, CEE were done by Camag. The HPLTC analysis to access to various compounds.

Sample loading

About 5µl Seed of each extracts are diluted with methanol and standard solution such as stigmaterol, quercetin, kameferol also were loaded as 6.0mm 60F 254 TLC plate by using Hamilton syringe and camag linomat instruments.

Photo documentation and Scanning

The developed TLC plate was dried to evaporate the solvent. Then TLC plate was kept in photo-documentation chamber (CAMAG Visualize) and the images were captured at white light, 254nm, 366nm and white light.

Finally the plate was scanning done by CAMAG TLC 366nm. The peak table, peak display were noted.

TEST: CEH, CEEA, CEET

Standard: Quercetin, kaempferol, Stigmaterol

Mobile phase: Toluene: Ethylacetate: Formicacid (6:4:0.1) [27-29].

2.9. Quantification of Flavonoids and Phenolic Compounds in Seed Extracts by Using LC-MS

LC Conditions

Mobile phase: 0.1% formic acid: methanol (45:65)

Column: phenomenex c18 column

Wavelength: 254nm

Flowrate: 0.8ml/min

Injvol: 20ul

MS Conditions

Ionization source: ESI

Analyser: single quadropole

Mobile phase: 0.1% formic acid: methanol (02:98)

Flowrate: 0.8ml/min

Injvol: 20ul

2.10. In vitro Antidiabetic Activity

2.10.1. Alpha Amyalase Inhibitory Assay

Alpha amylase is enzyme presnt in pancrease, its function are convert the polysaccharides into monasacharides such as glucose. glucose enter into the blood stream its leads to increase the post prandial hyperglycemia then its important tool for treatment of type two diabetes. The inhibition assay was illustrated by Miller. 1 mL of α-Amylase in 20mM phosphate buffer and 1 mL of plant extracts were added (Hexane, Ethanol and ethyl acetate extracts were prepared in 4 different concentrations i.e. 100µg/mL, 30µg/mL, 10µg/mL and 1 µg/mL) in test tubes. The whole reaction mixture was incubated at 25°C for 10 minutes. Then 1 mL of 1% Starch in 20mM phosphate buffer was added to the above mixture and incubated at 25°C for 10minutes. After

the incubation, 1 mL of DNSA reagent was added and incubated in boiling water bath for 5 minutes to stop the reaction. The solution was cooled to room temperature and 10 mL of distilled water was added for dilution. The absorbance was read at 540 nm. The OD values were taken at 540 nm for hexane, ethanol and ethyl acetate extracts for different concentrations and % Inhibition were mentioned below [38-40].

The inhibitory effect has been calculated by using the formula,

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} * 100$$

Control – DMSO + Enzyme + Substrate + DNSA

2.10.2 Glucose uptake Assay by using 3T3 L1 Cell Line.

Cell line and culture

3T3 cell line was acquired from NCCS, Pune. The cells were kept up in DMEM with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified environment of 50 µg/ml CO₂ at 37 °C. The 3T3L1 cells were cultivated at a cell thickness of 6 X 10⁴ cells/well at a last volume of 1000 µl in a 24 well plate with DMEM containing 10% FBS and hatched it for 48 hrs until the cells become intersecting.

Sample preparation

Take a 6 well plate and name it. Take 5ml of Solution A (Cell + Medium) and add in 6 well plate and Add 1ml of sample (Sample at selected dosage) to 5ml of solution A in the 6 well plate then incubate at 24hrs. After 24hrs, take the 6-well plate from the incubator and suck the entire medium from the plate. Take 2ml of 10XPBS and add to the 6-well plate for washing and suck out PBS and close the plate. Take 150µl of 10% SDS, add to the plates and shake well. Take a cell scraper and scrap the cells from the bottom of the plate and shift the cells into a corner of the plate. Take 150µl, take the total content from the plates and pour in eppendorf tube and named it separately. Allow to settle for 5 min. The 6 eppendorf tubes were placed in the centrifuge tube and. Then take pipette and take supernatant and add to labeled eppendorfs then Store in refrigerator 4°C for further use.

DNS Assay

Take the required no. of test tubes and label as follows to Take 200, 400, 600, 800, 1000 µl of glucose stock in each test tube. Make up the volume of 2ml with distilled water. Take 100µl of each sample supernatant in each test tube and make up a volume of 2ml with distilled water. Take 2ml of distilled water as a blank. Add 1ml of prepared DNS

reagent to all the tubes and Observe all the test tubes are of equal volume(3ml). Cover the entire test tube top with aluminum foil, Keep the test tubes with rack at 100°C in a water bath for 5 min., Observe the colour changes. Take 1ml of the solution and observe OD at 540nm.. Draw graph with amount of glucose in mg (mg/ml) as X axis and OD at 540nm as Y axis. Measure absorbance of the samples and detect the unknown concentration from the standard [41-45].

3. RESULTS

Table 1: Microscopic characters of *Casuarina equisetifolia*

S. No	Character	Observation
1	Size	6.5mm length, wide 3mm
2	Shape	Oval
3	Color	Brown
4	Odour	Aromatic
5	Texture	Smooth

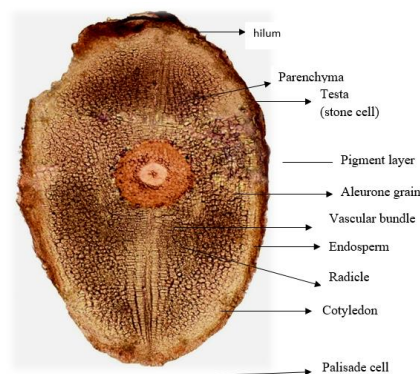
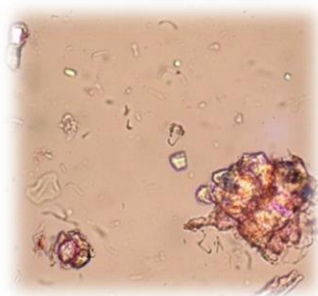
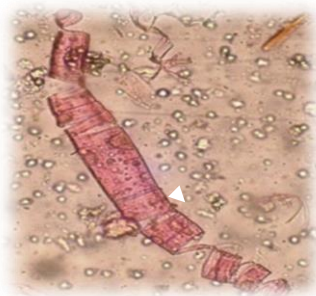


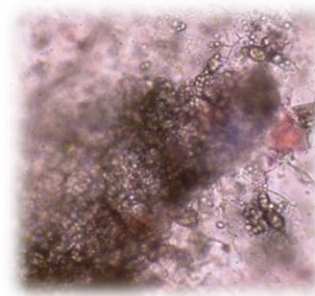
Figure 1: Scale measurement and morphology of *Casuarina equisetifolia* seed



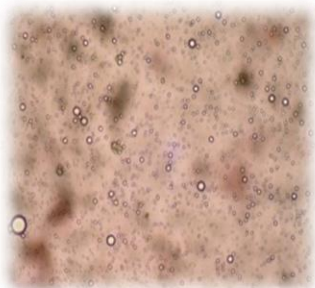
Prismatic calcium oxalate crystals



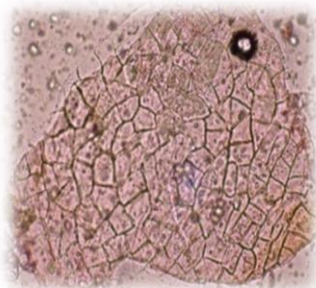
Spiral vessels



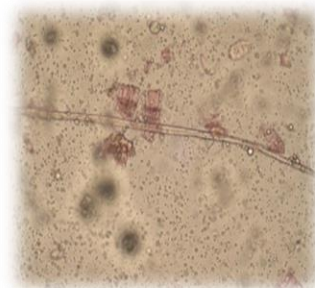
Fragment of cotyledon cells embedded with aleurone grain inflated with oil globules



Oil globules



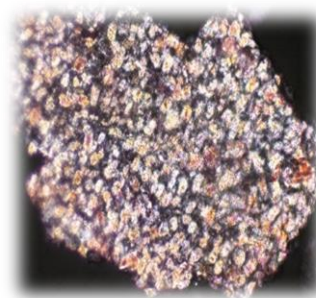
Cotyledons epidermis in surface view



Fibres



Transverly cut fragment of cotylden shows single layer of epidermal underline palsied parenchyma loaded with ol grains

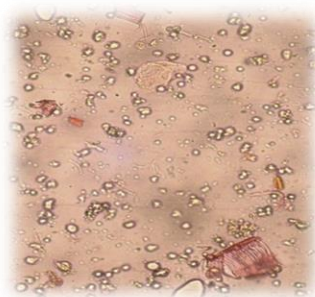


Testa under polarized light

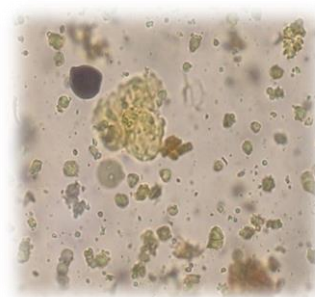
Figure 2: Transverse section of *Casuarina equisetifolia* seed



Porsan parenchyma



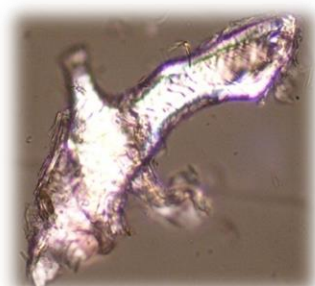
Starch grains



Starch grains dark blue, ol grains shows yellow colour



Scleroids



Scleroids under polarized light



Fragment of testa shows a layer of scleroids cells underline porsan parenchyma

Figure 3: Powder microscopy of *Casuarine equisetifolia* seed

Table 2: Physico chemical constants of *Casuarina equisetifolia*

S.NO	PARAMETERS	RESULTS (%)
1.	Total ash	
	Water soluble ash	1.6%
	Acid insoluble ash	0.7%
	Sulphated ash	5.7%
2	Water soluble extractive	11.0%
	Alcohol soluble extractive	17.10%
	Ether soluble extractive	1.4%
3	LOSS ON DRYING	6.07%
4	FOAMING INDEX	<100
5	SWELLING INDEX	8.5

Table 3: Percentage yield of successive extraction of *Casuarina equisetifolia*

S. No	Extraction	Method of Extraction	Physical Nature	Color	Percentage Yield(%W/W)
1	Hexane	Continuous hot percolation method using Soxhlet apparatus (successive solvent extraction)	semisolid	yellow	4.454%
2	Ethyl acetate		semisolid	green	1.19%
3	Ethanol		Sticky	black	4.92%

Table 4: Qualitative estimation of phytoconstituents

S.No	Chemical Constituents	Hexane Extract	Ethyl Acetate	Ethanol
1	Carbohydrates	-	-	+
2	Protein	-	+	+
3	Alkaloids	-	-	+
4	Phenolic	-	+	+
5	Tannins	-	-	+
6	Flavonoids	-	+	+
7	Glycosides	-	-	+
8	Sterols	+	+	+
9	Terpenoids	+	+	+
10	Volatile oils	-	=	+
11	Gums and mucilage	-	-	+
12	waxes	+	-	-

Note: + indicates presence, - indicates absence.

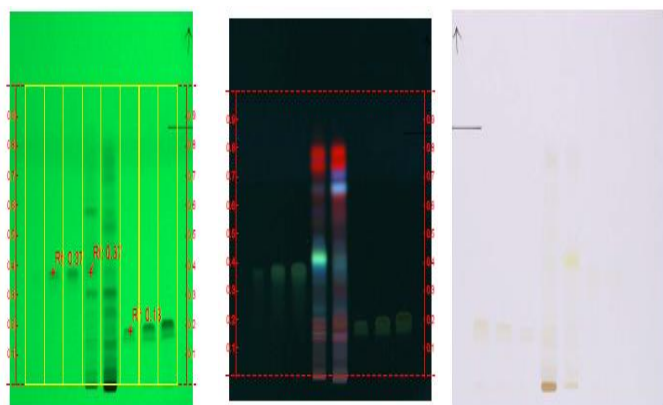


Figure (4-6): HPTLC Profile of quercetin, Kaempferol under 254, 366, And normal light in the various seed extracts of *Casuarina equisetifolia*

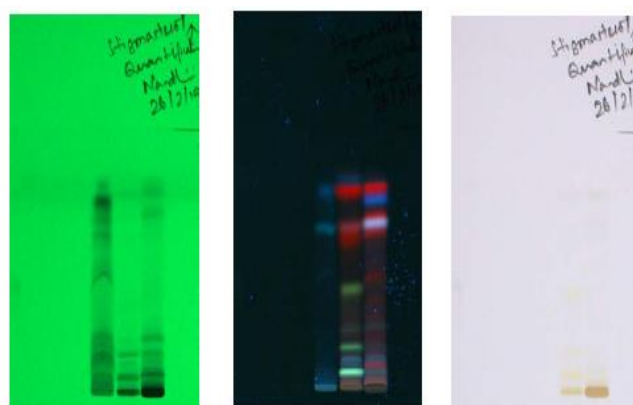


Figure (7-9): HPTLC Profile of stigmasterol under 254, 366, and normal light in the various seeds extracts of *Casuarina equisetifolia*

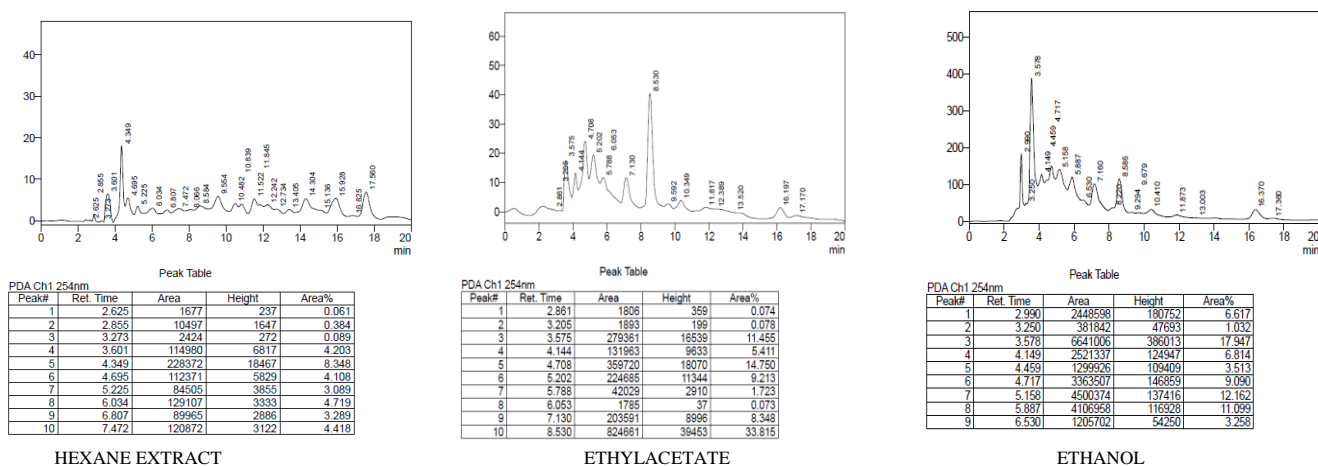


Figure (10-12): HPLC Quantification of Rutin and Ellagic Acid

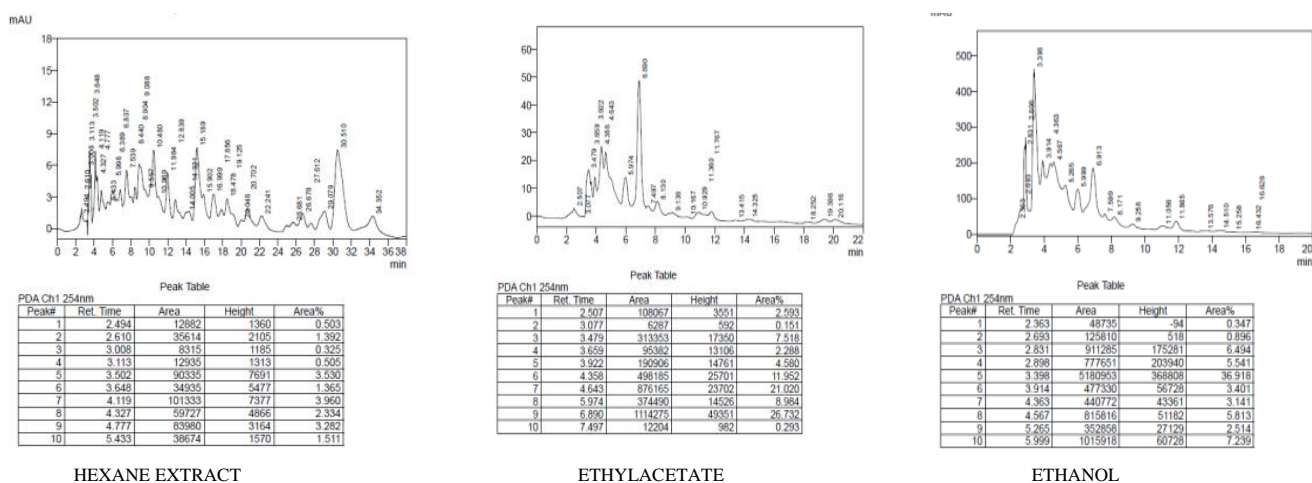


Figure (13-15): HPLC Quantification of Quercetin and Kaempferol

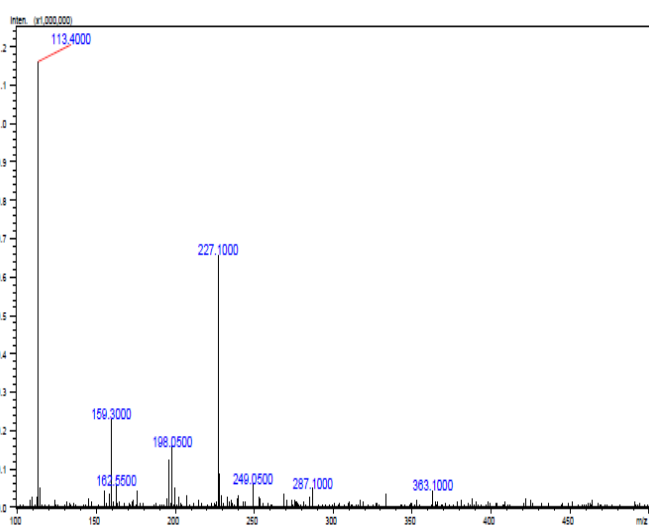


Figure 16: Mass Identification of Quercetin and Kaempferol

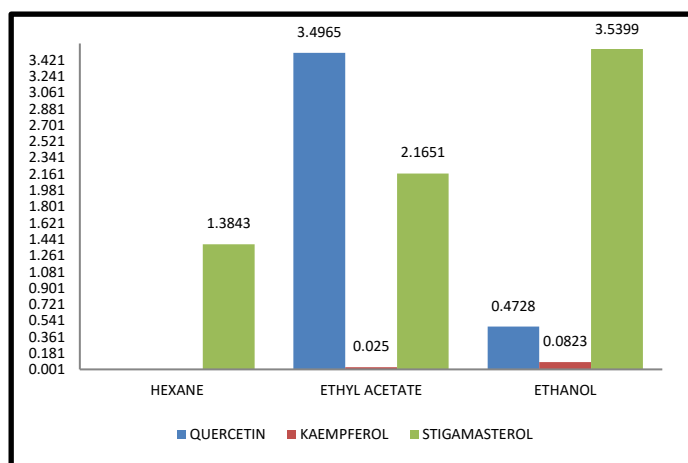


Figure 17: HPTLC quantification of various phytoconstituents of *Casuarina equisetifolia*

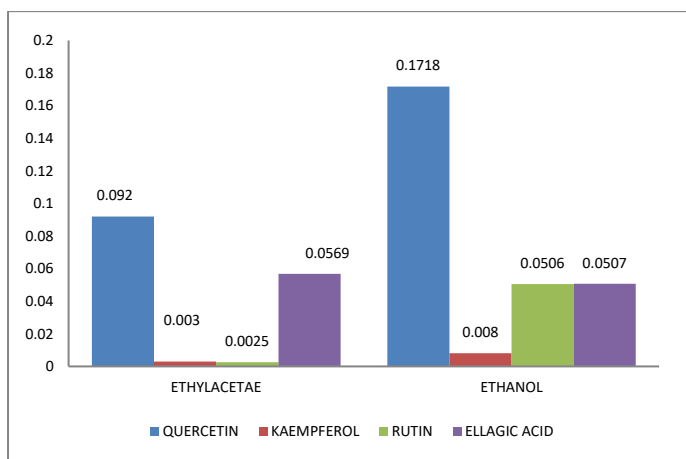


Figure 18: LC-MS quantification of various phytoconstituents from *Casuarina equisetifolia*

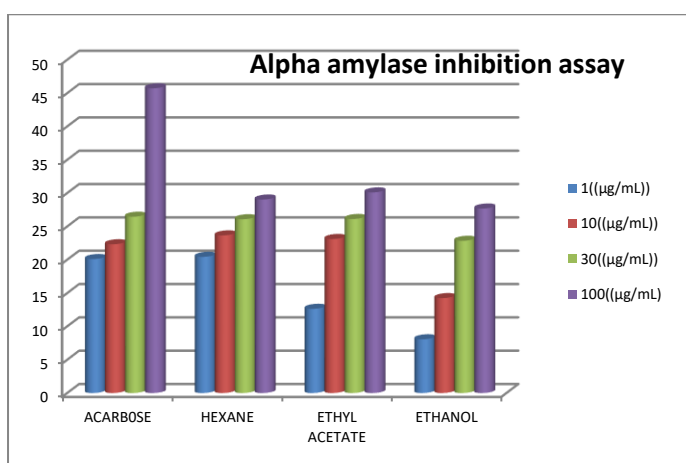


Figure 19: Alpha amylase inhibitory Assay of various plant extract of *Casuarina equisetifolia*

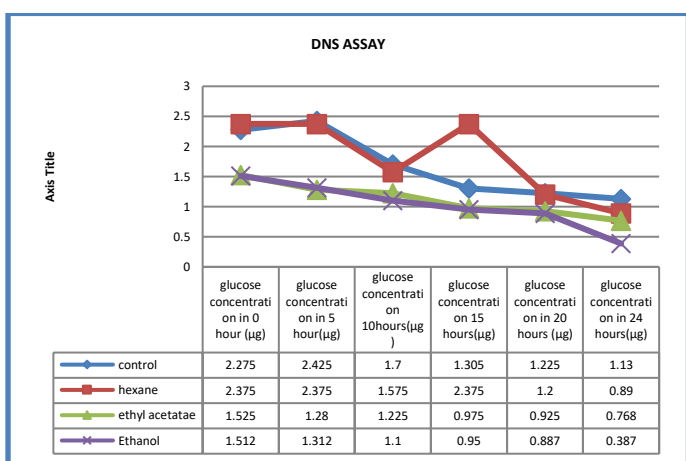


Figure 20: Glucose uptake assay of various plant extract from *Casuarina equisetifolia*

The seeds are 6.5-mm long with normal width of 3mm, smooth surface, oval-oval and packed fig No 1. Earthy colored or marginally dark in shading while sun-dried seeds are dull earthy colored hued having a smooth surface with smooth surface. Coarse powder of the seed is light earthy colored in shading with a fragrant scent Table No-1.

The powder microscopy revealed the presence of prismatic calcium oxalate crystals. Fragment of cotyledons cells are embedded with

Aleurone grains are flatted with oil globules. Transverly cutted fragment of cotyledons cells are show layer of epidermis underline it contain palisade parenchyma and loaded with oil globules. fragment of testa shows a layer of sclereids cells and its contain porsanparenchyma. Thesclereids are isodiametric and ressemble parenchyma cells so is called as stone cells are brachysclereids. It also contains starch grains, fibres, oilglobules. ThePowder characteristics of the seed have been shown in fig No 2, 3.

standardization such as total ash (5.0±0.21), water soluble ash (1.6±0.35) and acid insoluble ash (0.7±0.28) sulphated ash (5.7±0.53,) and loss on drying (6.07±0.32) were determined.

The extractive estimation of various concentrates was likewise determined they are ether dissolvable extractive was (1.4±0.16.) alcoholic extractive worth was (17.10 ±0.39) Water solvent extractive was (11.0±0.48w/w) were noted. Table No-2

The seed are subjected to the successive extraction by using solvent in increasing the polarity order such as Hexane Ethyl acetate, Ethanol then various types of active constituents are extracted from the coarsely powdered seeds *Casuarina equisetifolia* Linn. Successive extractive values reveald the solubility and polarity particulars the phytoconstituents in the seed. Percentages yield of various extracts were as follows, hexane (4.454% W/W), ethylacetate (1.19% W/W), ethanol (4.92% W/W). Ethanol Extract it shows highest extractive yield among the other extract. Table No-3. Preliminary Phytochemical analysis was performed by initially with different respective detecting reagents to the find out the type of the active constituents present in each extract such as Hexane, ethylacetate and Ethanol. The phytosterol triterpenoids are only present in the hexane extract. Ethyl acetate extract showed the presence of flavonoids alkaloids carbohydrates, saponins. All of the secondary metabolite is present in the high polar solvent such as ethanolic extraction Table No 4. The hexane separate comprises of stigmasterol (1.3843%w/w). The ethyl acetic acid derivation Concentrates comprises of Quercetin(3.4965% w/w) and Kaempferol(0.025% w/w) and stigmasterol(2.1651% w/w). The ethanol Concentrates comprises of Quercetin (0.4728% w/w), Kaempferol (0.0823% w/w) and stigmasterol(%3.5399w/w). The high convergence of Quercetin is available in ethyl acetic acid derivation separate among different concentrates. The high centralization of stigmasterol present in the ethanolic extricate as opposed to different concentrates Fig No-(4-9). The phytoconstituents such as Quercetin, kaempferol, rutin, ellagic acid were quantified by theLC-MS. The ethyl acetate extract consists of Quercetin (0.092% w/w), Kaempferol (0.003% w/w), rutin (0.0025% w/w) and ellagic acid (0.0569% w/w). The ethanolic extracts shows Quercetin (0.1718% w/w), Kaempferol (0.008% w/w), rutin (0.00506% w/w) and ellagic acid (0.0507% w/w). The ellagic acid is highly present in the ethyl acetate extract compare to other extracts. The high concentration of Quercetin and rutin is present in ethanol extract along with others. Fig No (10-15).The Quercetin and Kaempferol molecular peak was identified in ethylacetate, and ethanol extract by using Mass spectrometryFigNo-16. The in vitro anti-diabetic evaluation seed extracts are carried out by the different pharmacological studies such as Alpha amylase inhibitory assay And glucose uptake assay was carried out by 3T3L1 Cell line. Hexane extracts show maximum Alpha amylase inhibitory activity Fig No 19. Antidiabetic activity was accessed by the Aother method such as glucose uptake assay by using 3T3L1 cell line and glucose concentrations are determined by DNS method spectral analysis. The ethanol extract has greatest anti-diabetic activity due to the lower concentration of glucose. But in, ethyl acetate

and hexane extract has lesser anti-diabetic activity due to greater concentration of glucose. Fig No 20.

4. DISSCUSION

The ethnomedicinal use seed of *Casuarina equisetifolia* were been reported. The present study revealed the presence of quercetin, kaempferol, rutin, ellagic acid, this are active constituents present in the seed extracts of *Casuarina equisetifolia*. The in vivo antidiabetic activity of seed extracts of *Casuarina equisetifolia* they revealed the presence of significant anti diabetic activity. The ethanolic extract shows good anti diabetic activity when compared to other extracts. Further study is required for the molecular info of the extract behind this medicament activity. we have a tendency to will thus conclude from this study that the presence of the phytoche micals in these plants may well be the reason for the activity which the plants could primarily contain flavourer bioactive compounds that need additional structural elucidation and characterization methodologies to determine the bioactive constituents. additional investigations ought to be done for confirming the opposed diabetic activity of the plant. The plant extracts understudy will function therapeutic agents and might be used as potential sources of novel bioactive compounds for treating diabetes

5. CONCLUSION

The on top of experimental knowledge recommend that the ethanolic extract of tree equisetifolia seed possessed a major anti-diabetic property because it considerably increasing aldohexose uptake by victimization invitro technique. The antidiabetic drug activity may well be most likely thanks to the presence of flavonoids phytoconstituents gift within the extract. more studies ar needed to see the precise mechanism of action and to isolate and characterize the bioactive principles to blame for the claimed activity.

Affirmation

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