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PHARMACOLOGICAL AND PHYTOCHEMICAL SREENING OF IN-VITRO HEPATOPROTECTIVE ACTIVITY OF AAK (CALOTROPIS PROCERA)

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ABSTRACT

This article review comprises information regarding Phytochemistry and Pharmacological Activity of Aak (Calotropis Procera) -species of flowering shrubs belonging to family Apocynaceae.

Calotropis Procera also known as Aak, Apple of Sodom, Calotrope, Giant milkweed. This plant thrives in dry and arid regions , often found in wastelands, open fields, and along roadsides. This plant can survive in different types of soil such as sandy, rocky, and saline soils. It is found in various parts of Africa, (Sudan, Ethiopia, and Nigeria. It is also native to Asia, particularly India, Pakistan, and Bangladesh. In addition , it can also be found in Middle East (Saudi Arabia, Yemen, Oman). Cardenolides, steroids, tannins, glycosids, phenols, terpenoid, sugars, flavonoids, alkaloids and saponins are the major chemical constituents found in Calotropis Procera.

Calotropis Procera is mainly used as herbal medicine in treatment of various diseases such as body pain, asthma, epilepsy, cancer, snake bites, sexual disorders, skin diseases etc. Leaves, roots and latex of Calotropis Procera have been used in traditional medicine and believed to have antimicrobial, anti- inflammatory and analgesic properties. The silky fiber obtained from mature fruit follicles is used for making ropes, mats, and stuffing pillows. It is also planted in degraded areas to help stabilize soil and prevent erosion.

KEYWORDS: Calotropis Procera , Flavonoids, Antimicrobial, Alkaloids, Terpenoid, Epilepsy

INTRODUCTION

Calotropis procera (Sodom apple) is a member of the plant family Asclepiadaceae, a shrub about 6m high and is widely distributed in West Africa and other parts of the tropics [1]. The plant is erect, tall, large, much branched and perennial with milky latex throughout. In India, the secretions from the root bark are traditionally used for the treatment of skin diseases, enlargements of abdominal viscera and intestinal worms [2].

In Senegal, the milky latex is locally applied in the treatment of cutaneous diseases such as ringworm, syphilitic sores and leprosy. In Nigeria traditional medicine, C. procera is either used alone or with other herbs to treat common diseases such as fevers, rheumatism, indigestion, cold, eczema and diarrohea [3,4].

Calotropis procera (Aiton) is traditionally used to treat helminthiasis and dracunculiasis in India. It contains chemicals that might help thin mucus and make it easier to cough . In some animal study , calotropis has shown some activity against pain, inflammation, bacteria, fever, and ulcers caused by alcohol and medications such as aspirin, indomethacin (Indocin) etc.

Calotropis procera of family Asclepiadaceae is a tropical plant growing wild in warm climate up to an altitude of about 1050 meters. It is a native plant of North Africa. This plant is well distributed throughout India, particularly it is abundantly found in Rajasthan. It also found in Pakistan, Africa, Mexico, Australia, Egypt, Central and South America and Caribbean islands [6,7]. Latex of Calotropis procera is well known for cardiac glycosides and hydrocarbons [8]. The reported cardiac glycosides were Calotropogenin [9], Calotropin [10], Calotoxin, Uscharin and Calactin[11] with identification of some hydrocarbon derivatives like Linoleic acid, Oleic acid and Palmitic acid. The maximum portion of dry latex of C. procera extracted by acetone was 54%. During initial screening the other solvents tried were n-hexane, petroleum ether, chloroform and dimethyl sulphoxide but none of them could extract as much large part as acetone. Majority of the latex is composed of hydrocarbons therefore n-hexane part of acetone extract was concentrated for separation and identification.

Phytochemicals in Calotropis Procera

Phytochemical and biological activity screening is the primary steps for isolation of new biological active compounds, which lead to the discovery of new drugs.

To provide a scientific basis for traditional uses of Calotropis procera, the crude methanol extract of plant organs were investigated phytochemically. The plant contains various classes of bioactive secondary metabolites such as terpenoids, flavonoids, saponins, steroids and cardiac glycosides.

Importance of liver health

The importance of liver health cannot be overstated as the liver is a vital organ that performs a multitude of essential functions in the body. One of its key roles is metabolism and nutrient processing. The liver is responsible for metabolizing carbohydrates, proteins, and fats, ensuring their efficient utilization for energy production and the synthesis of important molecules. It helps regulate blood sugar levels, stores essential vitamins and minerals, and processes dietary substances to provide the body with the necessary building blocks for optimal functioning.[23]

Another critical function of the liver is detoxification and toxin removal. It filters and removes harmful substances, toxins, drugs, and metabolic byproducts from the bloodstream. The liver breaks down these substances into less harmful forms and facilitates their elimination from the body. Maintaining a healthy liver is crucial for effective detoxification, as a compromised liver can lead to the accumulation of toxins and increased risk of various health issues.[24]

The liver also plays a vital role in bile production, which aids in the digestion and absorption of fats. Bile helps break down fats into smaller molecules, allowing for their efficient absorption in the small intestine. A healthy liver ensures the production and proper flow of bile, supporting optimal digestion and nutrient absorption.[25]

Importance of heptoprotective activity

The importance of hepatoprotective activity lies in its ability to maintain and safeguard the health of the liver. The liver is a vital organ that performs numerous critical functions, including metabolism, detoxification, and synthesis of essential molecules. However, the liver is susceptible to damage from factors such as alcohol consumption, viral infections, medications, environmental toxins, and certain diseases.[31]

Hepatoprotective activity helps prevent and mitigate liver damage caused by these factors. Substances or interventions with hepatoprotective properties can help protect liver cells from injury, reduce inflammation, promote liver regeneration, and enhance the liver's ability to detoxify harmful substances. By preserving the structural integrity and functional capacity of the liver, hepatoprotective agents contribute to the overall health and well-being of an individual.[32]

Maintaining a healthy liver is essential for optimal digestion, metabolism, detoxification, and overall physiological balance. Hepatoprotective activity plays a crucial role in preserving liver health and preventing the development of liver diseases such as cirrhosis, hepatitis, and non-alcoholic fatty liver disease. Additionally, hepatoprotective interventions may also support the efficacy of certain medications by minimizing liver toxicity and side effects.[33]

Adopting a healthy lifestyle, including regular exercise, a balanced diet, limited alcohol consumption, and avoiding exposure to harmful substances, is fundamental for liver health. Additionally, certain natural compounds, such as silymarin (from milk thistle), curcumin (from turmeric), and antioxidants, have shown hepatoprotective effects. However, it is essential to consult a healthcare professional for guidance on appropriate hepatoprotective strategies, especially in cases of existing liver conditions or specific medical circumstances.[34]

Mechanisms of Hepatic Damage

Oxidative stress and free radical damage

Oxidative stress and free radical damage are key mechanisms involved in hepatic damage. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system in the liver. ROS are highly reactive molecules that can cause damage to cellular structures, including lipids, proteins, and DNA, through a process known as oxidative damage.[35]

In the liver, oxidative stress can arise from various sources, including the metabolism of drugs and toxins, alcohol consumption, viral infections, and certain diseases. These factors can lead to an overproduction of ROS or a reduction in the liver's antioxidant defense mechanisms, resulting in increased oxidative stress.[36]

The liver has a limited capacity to neutralize ROS, and excessive oxidative stress can lead to damage to liver cells. ROS can initiate lipid peroxidation, a process that damages cell membranes and disrupts their integrity. This can lead to the release of pro-inflammatory molecules and trigger an inflammatory response in the liver.[37]

The generation of ROS and oxidative stress can also impair the liver's detoxification function. The liver is responsible for metabolizing and eliminating various toxins and drugs from the body. However, excessive oxidative stress can lead to the depletion of cellular antioxidants and enzymes involved in detoxification processes, compromising the liver's ability to eliminate these harmful substances.[38]

Drug-induced hepatotoxicity

Drug-induced hepatotoxicity refers to liver damage or injury caused by the use of medications or drugs. Hepatotoxicity can range from mild elevation of liver enzymes to severe liver failure. There are several mechanisms through which drugs can cause liver damage:[39-42]

Direct Toxicity: Some drugs have inherent toxic effects on liver cells. They can directly damage hepatocytes (liver cells), leading to cell death and inflammation. Examples of drugs known for their direct hepatotoxicity include acetaminophen (overdose), certain antibiotics (such as isoniazid and rifampicin), and some chemotherapy drugs.[43]

Metabolic Activation: Certain drugs undergo metabolic activation in the liver, producing toxic intermediates that can damage liver cells. For example, drugs like valproic acid and certain antituberculosis medications require metabolic activation in the liver, and this process can lead to the generation of reactive metabolites that cause

hepatocellular injury.[44]

Immune-Mediated Reaction: In some cases, the body's immune system can recognize drugs or their metabolites as foreign substances, triggering an immune response that damages liver cells. This immune-mediated reaction can result in liver inflammation and injury. Drugs associated with immune-mediated hepatotoxicity include certain antibiotics (such as amoxicillin-clavulanate), anti-seizure medications (e.g., phenytoin), and some non-steroidal anti-inflammatory drugs (NSAIDs).[45]

Cholestasis: Some drugs can interfere with the normal flow of bile, resulting in a condition called cholestasis. Cholestasis impairs the excretion of bile from the liver, leading to the accumulation of bile acids and other substances, which can be toxic to liver cells. Drugs that can cause cholestasis-induced hepatotoxicity include certain antibiotics (such as erythromycin and azithromycin), anabolic steroids, and some hormonal contraceptives.[46]

Drug-Induced Fatty Liver: Certain medications can cause an accumulation of fat in the liver, leading to fatty liver disease. This condition is associated with liver inflammation and can progress to more severe forms of liver damage. Drugs known to contribute to drug-induced fatty liver include corticosteroids, tamoxifen, and some antiretroviral medications used in the treatment of HIV.[47]

Alcohol-induced liver injury

Alcohol-induced liver injury, also known as alcoholic liver disease (ALD), is a progressive condition that results from chronic and excessive alcohol consumption. It encompasses a spectrum of liver diseases, including fatty liver (steatosis), alcoholic hepatitis, and alcoholic cirrhosis.[54]

The initial stage of ALD is fatty liver, or steatosis, which is characterized by the accumulation of fat within liver cells. Excessive alcohol consumption impairs the liver's ability to metabolize fats, leading to the buildup of fatty acids. Fatty liver is usually reversible if alcohol consumption is discontinued early.[55]

If alcohol abuse continues, the condition can progress to alcoholic hepatitis, which is characterized by liver inflammation and damage. Inflammatory cells infiltrate the liver, causing tissue injury and dysfunction. Symptoms of alcoholic hepatitis may include jaundice (yellowing of the skin and eyes), abdominal pain, fatigue, and liver enlargement. Alcoholic hepatitis can be severe and life-threatening, especially in individuals who continue to consume alcohol.[56-58]

If alcohol consumption persists, alcoholic hepatitis can progress to alcoholic cirrhosis. Cirrhosis is the irreversible scarring of liver tissue, which disrupts liver structure and function. Scar tissue replaces healthy liver tissue, leading to the loss of liver function and the development of complications such as portal hypertension, ascites (accumulation of fluid in the abdomen), hepatic encephalopathy (brain dysfunction due to liver failure), and an increased risk of liver cancer.[59-60]

MATERIAL & METHOD

Table 1 : Instrument List

INSTRUMENT	INSTRUMENT DETAIL
Round-bottom flask	Corning
Condenser	Chemglass
Heating mantle or hot plate	Cole-Parmer
Electric heater	Yamato Scientific
Digital pH meter	Mettler Toledo
Optical microscope	Zeiss
Filtration apparatus	Millipore Sigma

Rotary evaporator	Yamato Scientific
Refrigerator	Thermo Fisher Scientific
Digital balance	OHAUS
Magnetic stirrer	Corning
Vernier caliper	Mitutoyo
Graduated cylinder	Corning
Volumetric pipette	Corning
Test tubes	Corning

Table 2 : Material Used

Material Name	Company Name
Dried bark of Calotropis procera	Botanical garden
Methanol	Methanex Corporation
Sodium chloride	Morton Salt
Acetone	Honeywell
Ethanol	Valero Energy Corporation
Chloroform	Fisher Scientific
Folin-Ciocalteu reagent	Fisher Scientific
HCl	Fisher Scientific
DPPH, α-glucosidase	Fisher Scientific
Starch	Fisher Scientific
Ethanol	Fisher Scientific
Chloroform	Fisher Scientific
Other chemicals used were of analytical grade	

Plant Profile

Calotropis procera, commonly known as the Apple of Sodom or Milkweed tree, is a species of flowering plant in the family Apocynaceae. It is native to tropical and subtropical regions of Africa, Asia, and the Middle East.

Figure 1: Parts of Calotropis procera (stem, inflorescence, buds, leaves)

Botanical Name: Calotropis procera
Family Name : Apocynaceae
Common Name: Aak, Apple of Sodom, Calotrope, Giant milkweed
Part Used : Root, Flower, Latex, Root barks, Leaf, Whole plant

Morphology:

- Size: Calotropis procera is a medium-sized shrub or small tree that can grow up to 5-6 meters in height.
- Trunk and branches: The trunk is often crooked, with a thick and corky bark. The branches are thick and have a whitish appearance.
- Leaves: The leaves are large, simple, and alternate. They are oval or lanceolate in shape, with a leathery texture and a grayish-green color. The leaves have prominent veins and can reach a length of 10-20 cm.
- Flowers: The flowers are unique and attractive. They are star-shaped and have a diameter of about 5 cm. The color of the flowers varies from white to pink or lavender, with purple spots in the center. The flowers are fragrant and appear in clusters.
- Fruits: The fruits are large, woody follicles that are about 10 cm long. They are initially green but turn brown as they mature. Each follicle contains numerous seeds embedded in a silky, cotton-like fiber.

Distribution and Habitat:

- Calotropis procera is found in various parts of Africa, including countries like Sudan, Ethiopia, and Nigeria. It is also native to Asia, particularly the Indian subcontinent, including India, Pakistan, and Bangladesh. In addition, it occurs in the Middle East, including Saudi Arabia, Yemen, and Oman.
- The plant thrives in dry and arid regions, often found in wastelands, open fields, and along roadsides. It can tolerate a wide range of soil types, including sandy, rocky, and saline soils.

Uses

- Medicinal properties: Various parts of Calotropis procera, including the leaves, roots, and latex, have been used in traditional medicine. They are believed to have antimicrobial, anti-inflammatory, and analgesic properties.
- Fiber: The silky fiber obtained from the mature fruit follicles is used for making ropes, mats, and stuffing pillows.
- Land reclamation: Calotropis procera is sometimes planted in degraded areas to help stabilize the soil and prevent erosion.
- Ornamental plant: The attractive flowers of Calotropis procera make it a popular choice for ornamental purposes in some regions.

Collection

The bark of Calotropis procera was subjected to shade drying for about 1 week. The dried bark was further crused to powder was passed through the mess no.22 and stored in air tight container for further analysis.

Soxhlet Extraction

Grind the Calotropis procera into a fine powder to increase the surface area available for extraction. Ensure that the powder is homogeneous. Choose a suitable solvent for the extraction process. Common solvents used for Soxhlet extraction include ethanol, methanol, or a mixture of both. The choice of solvent depends on the specific compounds you are targeting for extraction. Assemble the Soxhlet apparatus, which consists of a round-bottom flask, a Soxhlet extractor, a condenser, and a collection flask. Place the black catechu seed powder in the Soxhlet extractor. Pour the chosen solvent into the round-bottom flask and heat it. As the solvent vaporizes, it rises into the condenser and drips onto the black catechu seed powder in the Soxhlet extractor. The

solvent gradually dissolves the target compounds from the seeds and gets collected in the round-bottom flask. The solvent in the round-bottom flask gradually fills up and reaches a siphoning level, causing it to flow back into the extraction chamber. This continuous extraction process allows for efficient extraction of the target compounds. The extraction process typically takes several hours, and it is often performed overnight. The duration can vary depending on factors such as the solvent used, the sample size, and the desired level of extraction efficiency. Once the extraction is complete, the collected extract in the round-bottom flask is concentrated. This can be done by evaporating the solvent using techniques such as rotary evaporation or vacuum drying.

The % Yield in different solvents plant extracts were calculated by using the following formula:

% Yield = (Net weight of powder in gram after extraction /Total weight of powder in gram taken for extraction) ×100

Figure-2 : Soxhlet appratus

Physicochemical Evaluation

Physicochemical evaluation is a broad term that encompasses various analytical tests and measurements performed to assess the physical and chemical properties of a substance or material.

These evaluations provide valuable information about the composition, structure, and characteristics of a sample.

While the specific tests conducted can vary depending on the nature of the material being evaluated, here are some common physicochemical evaluations:

- **Loss on drying**

Mass loss as a percentage of mass/matter is referred to as loss on drying. A Petri plate containing 5–6g of medication powder is precisely weighed and stored in a hot-air oven with the temperature set at 105°C for 4-5 hours. Each case's weight loss was noted after chilling in a dessicator.

The process was repeated until the weight remained consistent.

Loss on drying (%) = loss in weight X 100/ W

W= weight of the drugs in grams.

- **Determination of Ash Value**

Ash value is a method for evaluating the purity and quality of unprocessed powdered pharmaceutical materials. Since the natural matter was burnt, which tells us more about the existence of the active ingredient in the unprocessed medicine, the debris is actually the rough material without the natural matter.

- **Total ash value**

Take 2 to 3g of precisely weighed powdered extract and

place it in a platinum or silica plate that has been tarred, lighted, and weighed. Disperse the medication powder on the plate's base. Increase the heat gradually, never reaching dull red heat, and incinerate until the material is free of carbon. Then let it cool and weigh it. If a carbon-free ash cannot be produced in this manner, the charred mass should be extinguished with hot water, the residue collected on an ashless filter paper, the filtrate added, the residue evaporated, and the residue ignited at a low temperature. First, an empty silica crucible was placed in the muffle furnace and heated to 600 °C for 30 minutes to ignite it. 2g of powdered medication was put to the silica crucible after it had been removed and weighed. After that, you put it in a muffle furnace for a couple of hours at 500–6000°C to make it white. finally weighed it. With an air-dried sample, the percentage of total ash was determined.

Total ash value = (z-x/y) × 100

Where, X = weight of the silica crucible
Y = weight of the drug powder (g)
Z = weight of the silica cruicible with powder ash

- **Acid-insoluble ash**

The insoluble material was removed from the ash after it had been heated for 10 to 15 minutes with around 30 millilitres of diluted hydrochloric acid. It was lit, cleaned with hot water, and weighed. In order to quantify the proportion of acid-insoluble ash, the air-dried medication was used as a reference. To 25 ml of dil HCl, the ash produced by the aforementioned technique was added. For five minutes, boil it. The residue was then collected on filter paper with less ash. Then, it was heated to 560^0C for 1 hour in a muffle furnace. With reference to the sample that had been air dried, the percentage of acid-insoluble ash was estimated.

Acid insoluble ash value % = (A/Y) × 100

where, A = weight of the remaining residue
Y = weight of crude powder taken (g)

- **Water-soluble ash**

The ash from the total ash value was boiled in 25 cc of water for 5 minutes. The insoluble material was added to filter paper with no ash. Then, a low temperature ignition was used to maintain a steady weight. By deducting the weight of water insoluble ash from the overall ash value, the weight of water soluble ash was calculated. Calculations were used to determine the amount of water-soluble ash in relation to air-died value.

- **Extractive Value**

Procedure 100 ml of solvent (chloroform, ethanol, and water) was macerated with 5 gm of coarsely powdered, air-dried medication for 24 hours, shaking regularly for 6 hours, and allowing to stand for 18 hours. After that, it was quickly filtered to prevent alcohol loss. A 25 ml sample of the filtrate was dried to dryness in a shallow dish with a flat bottom, dried at 1050°C, and weighed. The proportion of extractive that is soluble in alcohol was estimated using the air-dried medication as a base. The amount of soluble components needed for extraction in that particular solvent is referred to as the extractive value.

Extractive value was determined using the formula

$$\text{Extractive value (\%)} = \frac{\text{weightofresidue}}{\text{weightofdrypowder}} \text{ x } 100$$

- **Phytochemical Screening**

Standard procedures were followed for the phytochemical analysis of the powdered seed and/or aqueous seed extract (made by Soxhlet extraction). The next sections give a quick explanation of the methodologies. Three copies of every analysis were performed.

For the qualitative analysis of alkaloids, carbohydrates, fixed oils, flavonoids, glycosides, phytosterol/terpenoids, saponins, and tannins/phenols, preliminary phytochemical screening was carried out following the prescribed methods. the following screening exams:

- **Detection of alkaloids**

The residue obtained from the evaporation of 50mL of the alcoholic extract was titrated with 20mL of dilute hydrochloric acid and 0.5 g of sodium chloride and filtered. The filtrate was rendered alkaline with ammonium hydroxide and then extracted with successive portions of chloroform. The combined chloroform extract evaporated to dryness, the residues dissolved in 2mL hydrochloric acid and tested with silicotungestic acid and Mayer's reagents.

The formed precipitate was, in each case, indicates the presence of primary, secondary and tertiary alkaloids. The aqueous alkaline layer was acidified with hydrochloric acid and tested with silicotungestic acid and Mayer's reagents. A precipitate was formed indicates the presence of quaternary alkaloids.

- **Detection of flavonoids and anthocyanidins**

About 50mL of alcoholic extract was evaporated to dryness. The residue was titrated with 15mL of petroleum ether (60–80◦C) while warming. The residue was filtered and re-extracted again in the same manner. The defatted residue was titrated with 50mL of 80% methanol while warming then filtered. To 2mL of the filtrate, 0.5mL of hydrochloric acid was added and the mixture was warmed on a steam bath for 5 min, a red-violet colour, indicates the presence of leuco anthocyanidins. To another 2mL of the filtrate Shinoda test for flavonoids was applied. A red colour observed within 10 min indicates the presence of flavonoid compounds.

- **Detection of unsaturated sterols and triterpenoids**

The petroleum ether extracts, which were collected from flavonoid test, were evaporated to dryness. The residue was dissolved in 10mL choroform, dried over anhydrous sodium sulfate and filtered. The filtrate was divided into three portions.

The first portion was subjected to Liebermann-Burchard test; a blue green colour indicated a positive test for sterols while red-pink or violet indicate a positive test for triterpenoids. The second portion was subjected to Salkowski test; a red colour

indicates a positive test for sterol. The third portion was used as control for colour changes.

- **Detection of saponins**
  About 1 g of the dried powdered organs was macerated with 4mL of water, filtered, and the filtrate was shaken vigorously. A persisting froth for about 30 min was formed, indicating the possible presence of saponins. 5 mL of the alcoholic extract was evaporated to dryness under vacuum and the residue was dissolved in 10mL of normal saline. To 8mL of this solution 2mL of de-fibrinated blood in normal saline (1 : 40) were added and left for 24 hours. Blood haemolysis was noticed, indicating the presence of saponins.
- **Detection of coumarins**
  About 50mL of the hydroalcoholic extract was concentrated to 5mL and then treated with 25mL 10% alcoholic potassium hydroxide at room temperature. After standing for 30 minutes with occasional shaking, it was extracted with chloroform (4×25 mL).
  The aqueous layer was acidified with 10mL of 10% hydrochloric acid and refluxed for 1 hour. After cooling, it was extracted with chloroform, washed with water and dried over anhydrous sodium sulphate. The chloroform extract was evaporated to dryness. The residue was sublimed at 100◦C for 1 hour under vacuum. The sublimate was dissolved in 10mL spectroscopic alcohol and measured in the UV region 250–350 nm. The absorption in this region was taken as an evidence for presence of coumarin.

- **Detection of anthraqinones**
  The alcoholic extract corresponding to 5 g of each plant material was shaken with 10mL benzene
  and filtered. About 5mL of 10% ammonium hydroxide solution was added to the filtrate, shaken and allowed to stand until the two layers were separated.
  The development of pink, to violet colour in the ammonical phase indicates the presence of free anthraquinones. About 5 g of each plant extract was boiled with 10mL of 1% aqueous sulphuric acid and filtered while hot, the filtrate was shaken with 5mL benzene. The benzene layer was removed and shaken with 5mL of 10% ammonia solution.
- **Detection of tannins**
  About 20mL of alcoholic extract was evaporated under vacuum (temperature not more than 35◦C). The residue was stirred with 10mL of distilled water and filtered. On the addition of ferric chloride reagent to a portion of the filtrate, the formation of a green blue to bluish black colour or precipitate may indicate the presence of tannins.
- **Detection of cardiac glycosides**
  The 80% alcoholic extract remaining after the flavonoid test was divided to three portions. 5mL of the solution were placed in small porcelain evaporating dish, 5mL of kedde's reagent, and 5mL of 2 N sodium hydroxide solution were added. The appearance of purple colour indicates a positive test for cardiac glycosides. Another 10mL of the solution were evaporated to dryness, the residue was triturated with 3mL of ferric chloride solution and filtered. The filtrate was transferred to a test tube and 1mL of concentrated sulphuric acid was added slowly down the side of the test tube.
  The appearance of purple ring indicates presence of cardiac glycosides (desoxysugar). If the above two tests are positive, 5mL of the solution were evaporated to dryness. The residue was dissolved in 2mL chloroform and transferred to a small test tube. Acetic anhydride (0.3 mL) was added and mixed gently, then, a drop of concentrated sulphuric acid was added. The appearance of blue-green colour, observed during 60 min, indicates presence of cardiac glycosides (as steroids).
- **Cell Culture**
  HepG2 cells were maintained at 37°C in a 5% CO2 humidified incubator. Cells were cultured in Dulbecco's modified Eagle's medium enriched with 10% Fetal bovine serum and 1% penicillin and streptomycin. The culture media were changed every 2 days and subcultured when cells reached about 90% confluency. When the cells reached 80–90% confluence, they were trypsinized and plated at 30,000 cells per well in a 96-well microplate, 1 × 106 cells per well in six plates, or 5 × 107 cells per well in a single dish, depending on the determination. The cells were used after attachment.
- **Evaluation of Cell Viability**
  The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay provides a sensitive determination of the metabolic status of cellular mitochondrial enzymes. We did MTT assay according to a previously described method with slight modification. HepG2 cells (1 × 104 cells/well in 96 well plates) were cultured at 37°C for 24 h with 10% FBS prior to experimental treatment. For evaluating the cytotoxicity of GK extract, cells were treated with GK (1, 5, 10, 20, and 40 μg/mL) for 24 h. In contrast, measuring the cell viability, cells were pretreated for 1 h with different concentration of GK (5, 10, and 20 μg/mL) in FBS-free media and then cotreated with GK and NaAsO2 (10 μM) for an additional 24 h. The culture medium was replaced by 0.5 mg/mL MTT solution and cultured for another 2 h. MTT solution was carefully aspirated and blue formazan solubilized in DMSO. OD was measured at a wavelength of 570 nm using a tunable versa max microplate reader (Molecular Device, USA).
- **Measurement of Intracellular ROS and LDH**
  Intracellular ROS (Reactive oxygen species) generation and LDH (Lactate dehydrogenase) release into the culture medium by dead cells were determined using the ROS detection kit and LDH cytotoxicity assay kit, according to manufacturer instructions. HepG2 cells (1 × 104 cells/well in 96 well plates) were cultured at 37°C for 24 h. After adherence, cells were pretreated with GK (5, 10, and 20 μg/mL) for 1 h and followed by cotreatment with 10 μM NaAsO2 and GK for another 24 h. Absorbance was measured at 490 nm using a tunable versa max microplate reader.

RESULT

Physical Test of Crude Drugs

Insights on the nature, colour, odour, and taste of Calotropis procera extract can be gained from knowledge of its physical features in their basic pharmacological form. The Calotropis procera extract physical test findings are as follows:

Table 3 : The Organoleptic properties of the plant extract were evaluated for Calotropis procera extract

S.no	Parameter	Result
1.	Odour	Unpleasant
2.	Colour	Light to dark brown in color
3.	Texture	Thick and fleshy
4.	Taste	Bitter taste
6.	Solubility	Ethanol or Methanol

Extractive Values

Table 4 : The Extractive Values of the plant extract were evaluated for alcoholic and aqueous solutions Calotropis procera Extract

Crude drugs	Methanol% w/w	Ethanol % w/w
Calotropis procera Extract	15.50	12.20

Graph of the Extractive Values

The extractive values provide a quantitative measure of the solubility and extractability of various chemical compounds present in the plant material. These values can be useful in determining the quality and potency of the extract and can be used as a basis for standardization and quality control in herbal medicine or other applications where the extract is utilized.

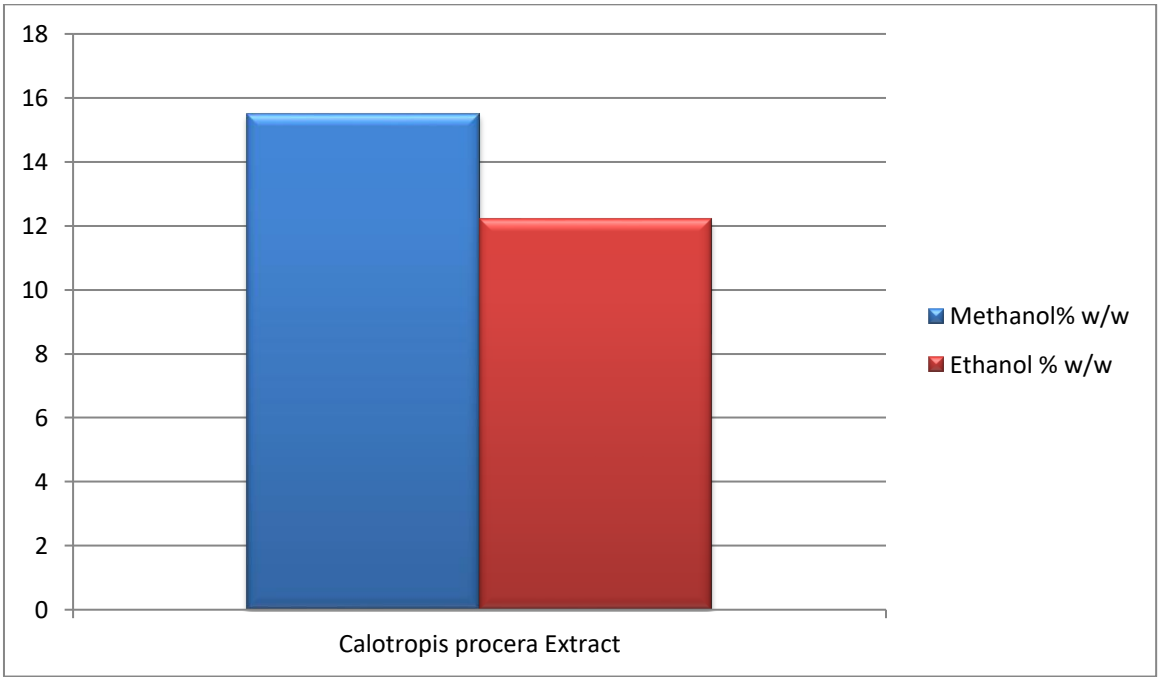


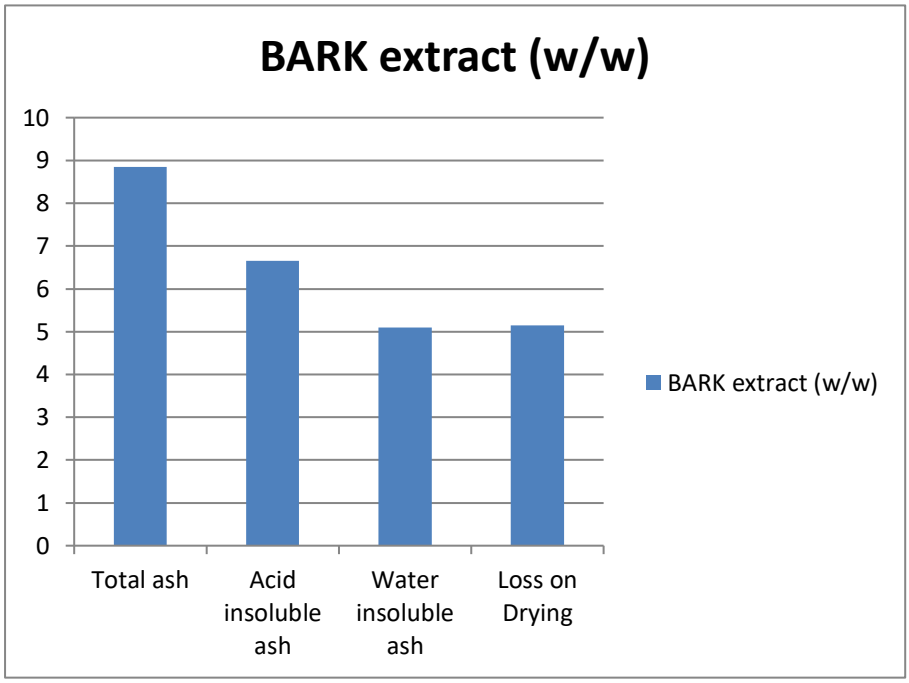
Table 5 : Loss on Drying And Foreign Organic Matter Calotropis procera extract

S.No	Physical Constant	Values (% W/W)
1.	Moisture content	5.21
2.	Foreign matter	2.25
3.	Extractive value(%w/w)	16.05

Table 6 : Total Ash, Acid Insoluble Ash And Water Soluble Ash Values Calotropis procera extract

S.no	Properties	BARK extract (w/w)
1.	Total ash	8.85
2.	Acid insoluble ash	6.65
3.	Water insoluble ash	5.10
4.	Loss on Drying	5.15

Table 7 : Bark Extract (w/w)



Phytochemical Screening

Phytochemical screening was performed on the fraction of the dry extract using the technique developed by Trease, Evans, and Harbourne. Alkaloids, saponins, glycosides, proteins, phytosterols, flavonoids, triterpenoids, tannins, fixed oil, and fats were all tested for throughout the phytochemical screening. Below is a list of outcomes:

Table 8 : Phytochemical screening for extract of Calotropis procera Extract

S.No	Chemical Tests	Calotropis procera Extract
1.	Tests for Steroids and Triterpenoids:	
	• Liebermann's Burchard Test	-
	• Salkowski Test	-
2.	Test for Saponins:	
	• Foam Test	+
3.	Tests for Alkaloids:	
	• Hager's Test	+
	• Mayer's Test	+
4.	Tests for Glycosides:	
	• Borntrager's Test	+

	• Keller Killiani Test	+
5.	Tests for Tannins and Phenolic compounds:	
	• Gelatin Test	-
	• Ferric Chloride Test	-
	• Lead Acetate Test	-
	• Dilute Nitric acid Test	-
6.	Tests for Flavonoids:	
	• Ferric chloride Test	+
	• Alkaline reagent Test	-
	• Lead acetate Test	-
7.	Tests for Proteins:	
	• Biuret Test	+
	• Xanthoproteic Test	-
8.	Test for Carbohydrates:	
	• Fehling Test	-

" + " Found " – " Not Found

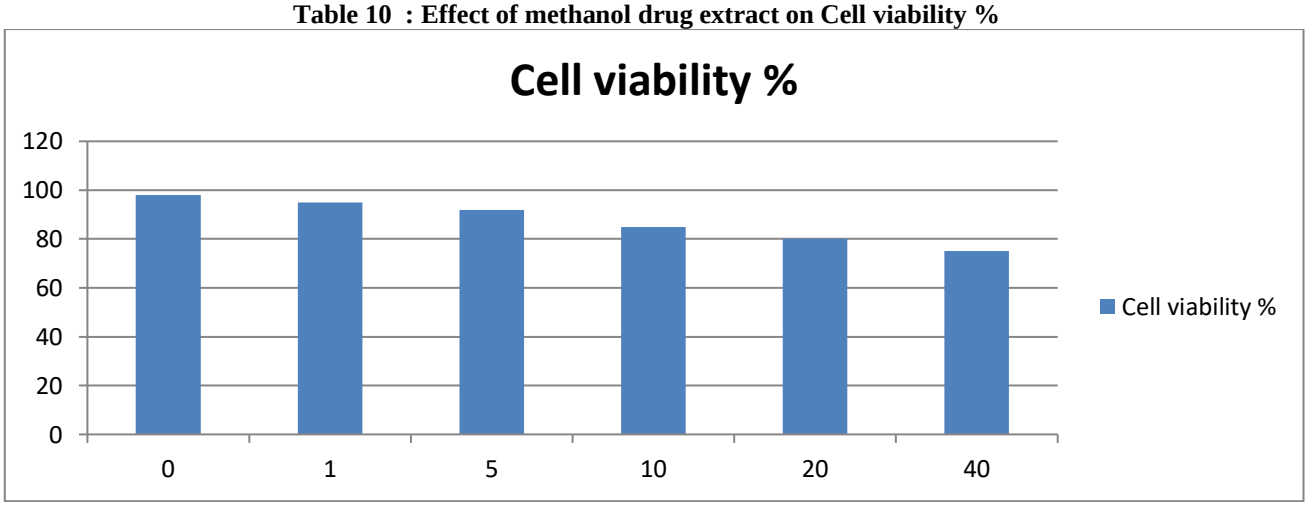
Reduced Na As O2 - Induced Cytotoxicity in Hep G2 Cells

We performed MTT assay to evaluate the mechanism responsible for the hepatoprotective effects of Calotropis procera extract against Na As O2-induced damage in Hep G2 cells. To investigate the cytotoxicity of Calotropis procera extract, the MTT assay was done using a different concentration of Calotropis procera extract (1, 5, 10, 20, and 40 µg/mL) for 24 h. The table shows that as the concentration of Calotropis procera extract increases, the cell viability percentage decreases. At a concentration of 0 µg/ml, the cell viability is 98%. However, as the concentration increases to 1 µg/ml, the cell viability decreases to 95%. This trend continues with further increases in concentration, resulting in a gradual decrease in cell viability percentage. At the highest concentration tested (40 µg/ml), the cell viability is 75%.

Table 9 : Effect of methanol drug extract on Cell viability %

S. No	Sample	Concentration (µg/ml)	Cell viability %
1.	Calotropis procera Extract -1	0	98
2.	Calotropis procera Extract -2	1	95
3.	Calotropis procera Extract -3	5	92
4.	Calotropis procera Extract -4	10	85
5.	Calotropis procera Extract -5	20	80
6.	Calotropis procera Extract -6	40	75

Table 10 : Effect of methanol drug extract on Cell viability %



Inhibited ROS Release in Hep G2 Cells

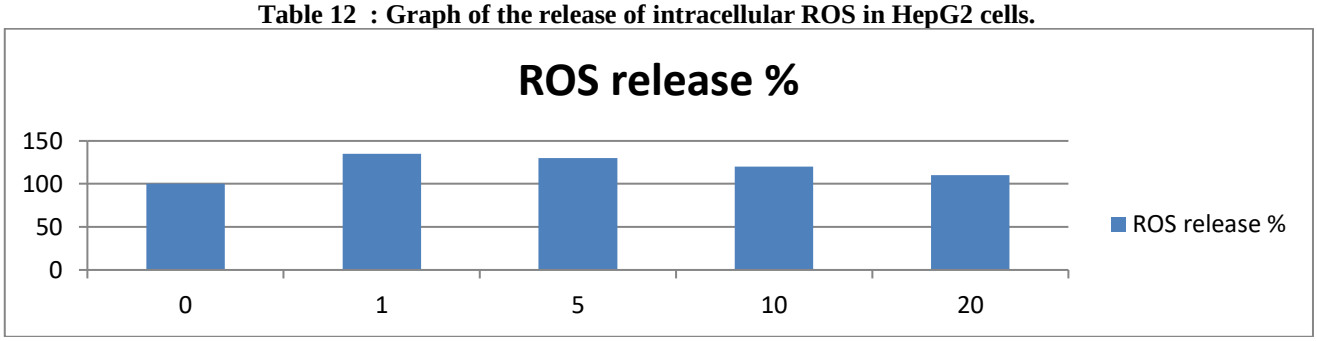
The table shows that as the concentration of Calotropis procera extract increases, the ROS release percentage generally increases. At a concentration of 0 µg/ml, the ROS release is 100%. However, as the concentration increases to 1 µg/ml, the ROS release increases to 135%. This trend continues with further increases in concentration, resulting in a gradual increase in ROS release percentage. At the highest concentration tested (20 µg/ml), the ROS release is 110%. It's important to note that an increase in ROS release may indicate oxidative stress or cellular damage.

Table 11 : Effect of methanol drug extract on ROS release (%)

S. No	Sample	Concentration (µg/ml)	ROS release %
1.	Calotropis procera Extract -1	0	100
2.	Calotropis procera Extract -2	1	135
3.	Calotropis procera Extract -3	5	130
4.	Calotropis procera Extract -4	10	120

5.	**Calotropis procera Extract -5**	**20**	**110**

Table 12 : Graph of the release of intracellular ROS in HepG2 cells.



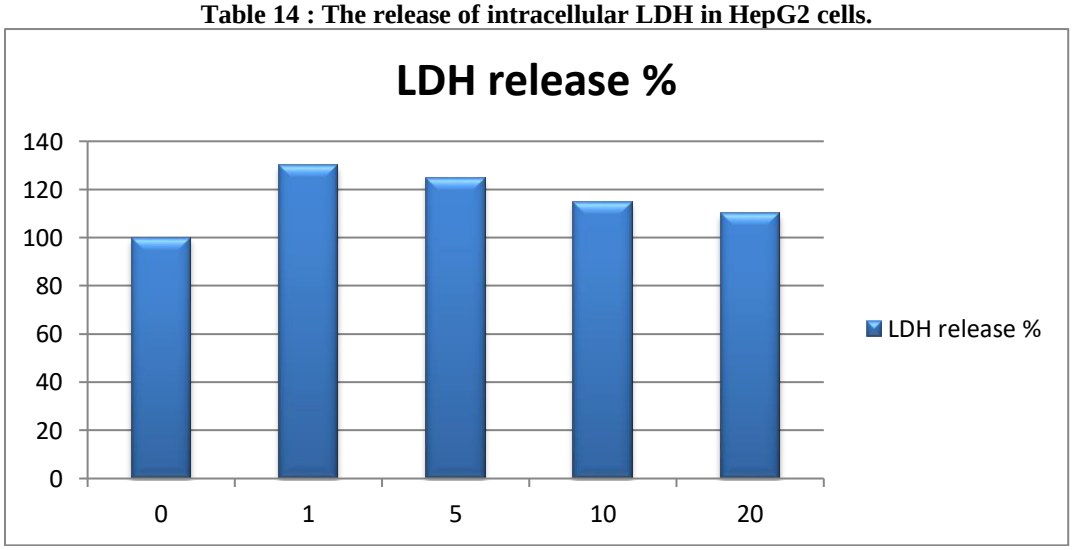
Inhibited LDH Release in Hep G2 Cells

The table shows that as the concentration of Calotropis procera extract increases, the LDH release percentage generally increases. At a concentration of 0 µg/ml, the LDH release is 100%. However, as the concentration increases to 1 µg/ml, the LDH release increases to 130%. This trend continues with further increases in concentration, resulting in a gradual increase in LDH release percentage. At the highest concentration tested (20 µg/ml), the LDH release is 110%. Increased LDH release can be an indicator of cellular damage or cytotoxicity.

Table 13 : Effect of methanol drug extract on LDH release(%)

S. No	Sample	Concentration (µg/ml)	LDH release %
1.	**Calotropis procera Extract -1**	**0**	**100**
2.	**Calotropis procera Extract -2**	**1**	**130**
3.	**Calotropis procera Extract -3**	**5**	**125**
4.	**Calotropis procera Extract -4**	**10**	**115**
5.	**Calotropis procera Extract -5**	**20**	**110**

Table 14 : The release of intracellular LDH in HepG2 cells.



CONCLUSION

- The substance being described has an unpleasant odour, a light to dark brown color, a thick and fleshy texture, a bitter taste, and can be dissolved in ethanol or methanol. No information is provided about the substance's powder form.
- The extractive values of Calotropis procera extract were determined to be 15.50% w/w in methanol and 12.20% w/w in ethanol. These values indicate the amount of the plant's constituents that were extracted in each respective solvent.
- The Calotropis procera extract has a moisture content of 5.21% w/w, foreign matter content of 2.25% w/w, and an extractive value of 16.05% w/w. These values provide information about the water content, presence of foreign material, and the efficiency of extraction for the extract.
- The Calotropis procera bark extract has a total ash content of 8.85% w/w, acid insoluble ash content of 6.65% w/w, water insoluble ash content of 5.10% w/w, and a loss on drying of 5.15% w/w. These values provide information about the inorganic content, insoluble residues, and moisture content of the extract.
- The phytochemical screening of Calotropis procera extract revealed the presence of saponins, alkaloids, glycosides, flavonoids, and proteins. However, steroids, triterpenoids, tannins, phenolic compounds, and carbohydrates were not detected in the extract. The specific tests used and their corresponding results are summarized for each phytochemical category.
- The methanol extract of Calotropis procera exhibited a concentration-dependent effect on cell viability. At lower concentrations (0-5 µg/ml), the cell viability remained relatively high (ranging from 92% to 98%). However, as the concentration increased to 10-40 µg/ml, the cell viability decreased gradually, indicating a decrease in cell viability with higher concentrations of the extract.
- The methanol extract of Calotropis procera exhibited a concentration-dependent effect on ROS release. At a concentration of 0 µg/ml, the ROS release was 100%. However, as the concentration of the extract increased to 1-20 µg/ml, there was an increase in ROS release, indicating a higher level of reactive oxygen species being generated in the cells with higher concentrations of the extract.

- The methanol extract of Calotropis procera exhibited a concentration-dependent effect on LDH release. At a concentration of 0 µg/ml, the LDH release was 100%. However, as the concentration of the extract increased to 1-20 µg/ml, there was an increase in LDH release, indicating an increase in the leakage of LDH enzyme from the cells with higher concentrations of the extract.

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