

## Phytochemical Screening and Application of *Jatropha Curcas* Extract for the Development of Anti-Rabies Vaccine

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### ABSTRACT

Root and stem bark of the *Jatropha curcas* were collected from three areas of the Jos Plateau (Foron, Kuru and Bokokos) and air-dried for twenty-four weeks after which the dried samples were reduced to powder using stainless still pestle and mortar. Extracts of the reduced samples were then obtained using four solvent matrices, n-hexane, ethanol, benzene and acetone solvent systems respectively. Thirteen phytochemicals were identified from the extracts of the four different solvent matrices in which the test confirms the presence of various phytochemicals like flavonoids, saponins, steroids, glycosides and terpenoids, tannins, coumurins. The lowest yield was observed in n-hexane with 7%, while the highest yield was recorded in the benzene and acetone solvent systems with 43% and 46% respectively, indicating more phytochemicals in the benzene and acetone systems than that of n-hexane and ethanol systems. This work shows that benzene and acetone solvent systems are better choices for solvent extraction in plant sample matrix. A pilot model scheme for laboratory test application of the extracts for a possible vaccine development and treatment of rabies using mice as test animal was followed which gave a minimum inhibitory concentration of 0.2µg/ml indicating that the extracts can used to develop a potent vaccine or drug for the treatment of rabies.

Keywords: *Jatropha curcas*, Jos Plateau, Phytochemicals and Solvent Extraction Matrices.

### 1.0 INTRODUCTION

Rabies is an infectious viral disease that is almost always fatal following the onset of clinical signs. In up to 99% of human cases, the rabies virus is transmitted by domestic dogs (WHO, 2005, NASPHV, 2011). Rabies affects domestic and wild animals, and is spread to people through bites or scratches, usually via saliva. It is a neglected disease of poor and vulnerable populations whose deaths are rarely reported and where human vaccines and immunoglobulin are not readily available or accessible. It occurs mainly in remote rural communities where children between the ages of 5–14 years are frequent victims. Rabies is present on all continents with the exception of Antarctica, but more than 95% of human deaths occur in Asia and Africa (NASPHV, 2011).

*Jatropha curcas* is commonly called physic nut, purging nut or pig nut. Previous studies have reported that the plant exhibits bioactive activities for fever, mouth infections, jaundice, guinea worm, sores and join rheumatism (Oliver-Bever, 2000). *Jatropha curcas* is a plant which belongs to the family Euphorbiacea and originated from Mexico and South Africa (Tint and Mya, 2009).

*J. curcas* is a drought – resistant perennial plant, growing well in Marginal /poor soil. It is easy to establish and grows relatively quickly producing seeds for 50 years. *J. curcas* has limited natural vegetative propagation and is usually propagated by seed. The oil from *J.curcas* seeds is used to treat rashes and parasitic skin diseases. Mixture of oil from the seeds with benzyl benzoate is effective against scabies and dermatitis (Belewu, 2008). According to (Shama, (2013), the roots of *Jatropha curcas* Linn are traditionally used as anthelmintic, diuretic, anti-rheumatic, anti-pyretic, anti-convulsant, anti-hypertensive, anti-diarrheal, anti-inflammatory, in dysentery, jaundice and sore throat. They have been shown to possess anti-diarrheal, anti-inflammatory, antimicrobial, anti-oxidant and

molluscicidal activity. In spite of numerous medicinal uses there is a lack of data on the standards and pharmacognostical parameters regarding roots of the plant. So the study has been carried out in order to contribute to identification, maintain its quality, safety and reproducibility.

In a similar work reported by Medubi *et al* (2010), *Jatropha gossypifolia* abounds. The leaf decoction of *Jatropha gossypifolia* is used for bathing wounds and the stem sap used to stop bleeding and itching of cuts and scratches. Oduola *et al* reported that the raw extract of the leaf of *Jatropha gossypifolia* has anticoagulant activity and it was opined that if the active chemicals are isolated and purified the leaf extract could be used for therapeutic control of thrombosis. In some parts of Nigeria the root decoction in addition to salt is used to treat syphilis, general illness and gonorrhoea.

According to Sachdeva *et al* (2010) the plant extract showed a significant anti-diabetic activity comparable with that of glibenclamide, this research indicates that the *Jatropha curcas* stem bark possesses significant anti-diabetic activity. Similarly, a research conducted by Maksudur *et al* (2012) revealed that *Jatropha curcas* L. (Euphorbiaceae) is widely used throughout the world for various therapeutic properties. The roots of the plant also have numerous curative properties. However, Shama (2013), showed that there is a lack of data corresponding to the standardization and photochemical profile of roots of the plant and an infusion of the stem is taken to treat hypertension.

According to Protomedicine, the sap has a widespread reputation for healing wounds, as a haemostatic and for curing skin problems; it is applied externally to treat infected wounds, ulcers, cuts, abrasions, ringworm, eczema, dermatomycosis, scabies and venereal diseases. The sap has a styptic effect and is used against pains and bee and wasp stings. Dried and pulverized root bark is made into poultices and is taken internally to expel worms and to treat oedema.

The methanolic extract of the leaves of *Jatropha curcas* L. contains useful active ingredients which may serve as potential drug for the treatment of diseases (Ebuehi *et al*, 2009). They also reported that a combination of TLC, IRS and HPLC can be used to analyze and quantify the flavonoids present in the leaves of *Jatropha curcas* L. According to Oskoueian *et al* (2011) the extracts tended to scavenge the free radicals in the reduction of ferric ion ( $\text{Fe}^{+3}$ ) to ferrous ion ( $\text{Fe}^{+2}$ ). Cytotoxicity assay results indicated the potential of methanolic extract as a source of anticancer therapeutic agents toward breast cancer cells.

The antioxidant potential of phytochemicals is studied using the quenching of free radicals. The L. aquatica leaves and stem was further highlighted by the quenching of DPPH – free radicals, which is a proton free radical commonly used to determine the free radical scavenging power of antioxidants (Adesegun *et al.*, 2008). The decrease in absorbance of DPPH – I. aquatica extracts mixture in this study which measured the extent of radical scavenging activity of the extracts supported the findings of Asok-Kumar *et al.* (2009). In the present study, it was

observed that the stem extract exhibited better scavenging activity than the leaves. This observation is consistent with the findings of Huang *et al.* (2005). Similar results have also been reported by James *et al.* (2009). The stem extract had better scavenging activity ( $IC_{50} = 35.96 \mu\text{g/ml}$ ) than the leaves ( $IC_{50} = 176.92 \mu\text{g/ml}$ ). The higher scavenging activity of the stem may be as a result of the increased phenolic contents of the stem compared to the leaves. Olukemi *et al.* (2005) has reported a strong relationship between phenolic content and antioxidant activity in selected fruits and vegetables. Phenolic compounds are the major contributors to antioxidant activity (Hamid *et al.*, 2011; Zainol *et al.*, 2005).

In a research reported by Abhilash *et al* (2015), the Phytochemical screening revealed the presence of saponin, steroids, tannin, glycosides, alkaloids and flavonoids in the extracts, with the ability of the crude stem extracts of *J. curcas* to inhibit the growth of bacteria and fungi is an indication of its broad spectrum antimicrobial potential which may be employed in the management of microbial infections. Ahirrao, (2011) also reported that the moisture content of *Jatropha curcas* was found to be 1.70 %. In their work, preliminary phytochemical analysis test showed the presence of steroids, flavonoids, alkaloids, saponins, triterpenoids, tannins and carbohydrates. According to Villase *et al* (2011), the crude methanol extract of *Jatropha curcas* leaves exhibited activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium phlei*, *Candida albicans*, and *Trichophytonmentagrophytes* but was inactive against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*. Ekundayo and Ekekwa (2013) showed that the ethanolic extract of *J. curcas* does not show activity on most bacteriological infections.

Medubi *et al* (2010), gave the histological changes observed which are consistent with glomerulonephritis and include increased urinary (Bowman's) space, shrinkage and distortion of the glomerular tuft as well as scarring of the glomeruli, changes appear to be both dosage and time dependent and the administration of prednisolone as an adjunct did not exert any ameliorative effect.

They conclude that ethanolic root extract of *Jatropha gossypifolia* is toxic to the kidney and causes increased urea retention in the blood. *Jatropha curcas* shows scavenging effect against hydroxyl (OH), superoxide anion  $1-1+$  (O) and 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid radicalcation (ABTS) radicals. It has also been reported that *J. curcas* exhibits strong antimicrobial and antioxidant properties in all the in vitro assays (Sundari *et al* 2011).

Etinosa and Igbiosa (2011), reported a correlations between the amount of phenolic compounds and percentage inhibition of DPPH radicals scavenging activity of the extract ( $r= 0.98$ ). In their report, it was indicated that *J. curcas* is a potential source of natural antioxidants and may be a good candidate for pharmaceutical plant based products.

According to Iyalomh (2015), improved surveillance, availability and affordability of vaccines for pre- and post-exposure prophylaxis as well as interventions to prevent dog bite related injuries, particularly among children, are

imperative (Compendium of Animal Rabies Prevention and Control, 2011; National Association of State Public Health Veterinarians, Inc. (NASPHV) Recommendations and Reports November 4, 2011 / 60(RR06),1-14)

Experimental and historic evidence indicates that dogs, cats, and ferrets shed virus a few days before clinical onset and during illness. Clinical signs of rabies and include loss of appetite, dysphagia, cranial nerve deficits, abnormal behavior, ataxia, paralysis, altered vocalization, and seizures. Progression to death is rapid (National Association of State Public Health Veterinarians, 2011)

A Guide for Health Professionals Human rabies immune globulin (HRIG) is infiltrated around the site of the bite(s), and provides rapid passive immune protection with a half-life of approximately 21 days.

A research conducted by Maksudur R. Khan *et al* (2012) revealed that *Jatropha curcas* L. (Euphorbiaceae) is widely used throughout the world for various therapeutic properties. The roots of the plant also have numerous curative properties.

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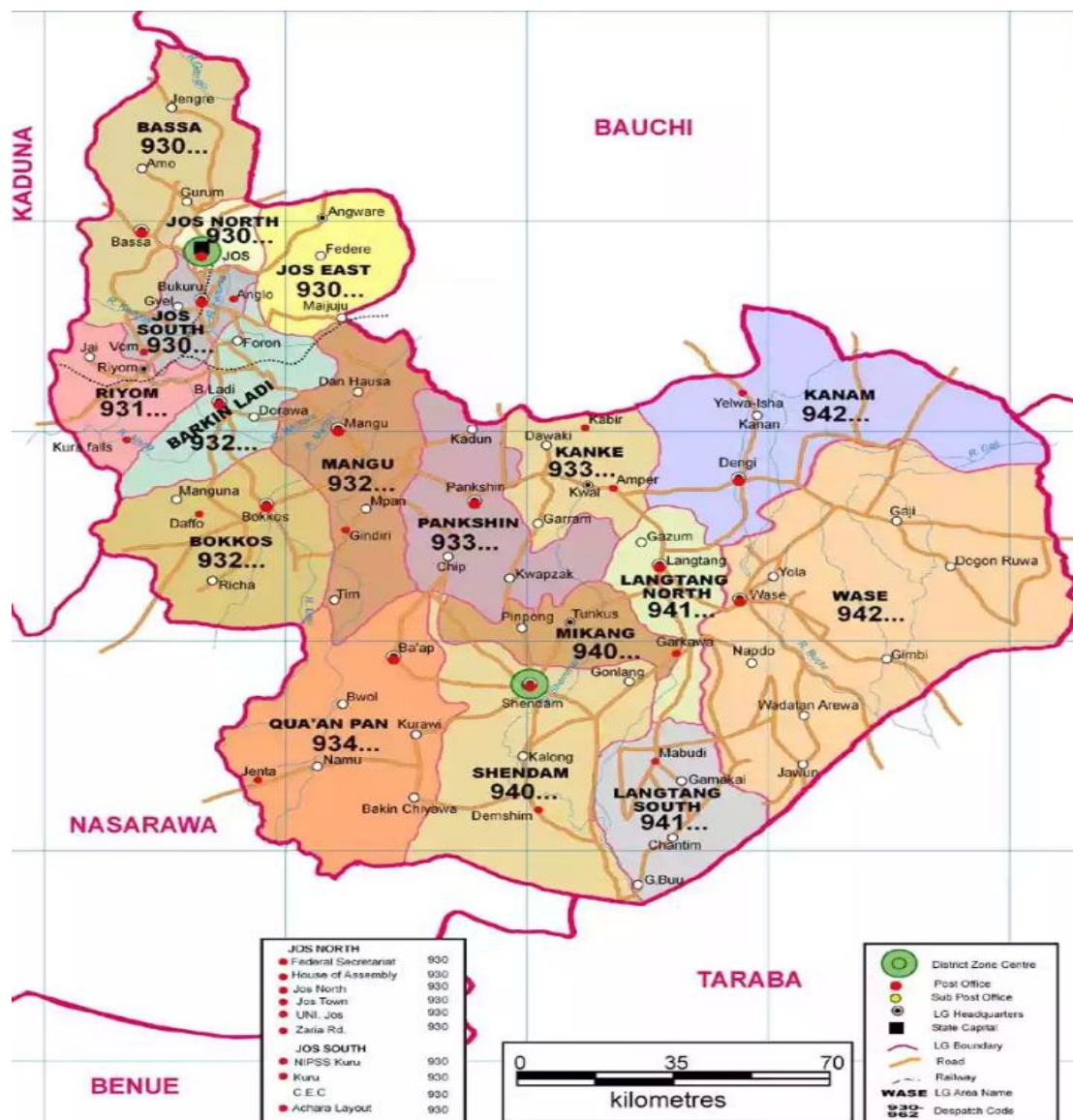
## **2.0 MATERIALS AND METHOD**

### **2.1 Description of sample areas**

Three sample areas, Foron, Kuru and Bokkos, all in plateau State, Nigeria were identified. These areas are all in the Northern Zone of the State situated almost at the highest altitude of the Jos Plateau.

### **2.2 Sample Collection**

*Jatropha curcas* stem bark were collected from a plantation in Foron, Kuru and Bokkos Local Governments of Plateau State, Nigeria in July 2015. The plant was identified by Dr. Nangbes Jacob and confirmed by Botany Department of Plateau State University.



**Fig: 3.1** Map of Plateau State

### 2.3 Sample Preparation

Preparation of plant materials: The stem bark and root were cleaned by hand to remove foreign materials. The stem bark and root were cut into small pieces prior to drying. It was dried under room temperature; the dried stem bark and root was then grounded using an electric grinder and stored in an air tight polyethene. The Powdered material was used further, for phytochemical screening.

### 2.4 Crude Extraction of Root and Stem bark samples of *Jatropha curcas*

The extraction of crude was carried out using a Soxhlet apparatus and various solvents in increasing order of polarity were applied. The solvents used for extraction were hexane, acetone, benzene and ethanol. 30.0 g portion grounded sample extract powder was wrapped in a thimble made of Whattmann filter paper and was placed into the main extraction unit of the Soxhlet apparatus. A round bottom flask containing solvent was placed on a heating mantle and temperature adjusted to the boiling point of each solvent. Once the solvent in the side arm became



colourless, the extraction process was stopped. The desired extract containing small amount of solvent was taken in a clean and dry pre-weighed petridish and the solvent was evaporated completely by distillation. The extracts were then stored in a cool and dry condition under the fume Cabot

## **2.5 Phytochemical Screening**

### **2.5.1 Test for Saponins:**

Aqueous extract of the solvent was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

### **2.5.2 Test for alkaloids.**

Each extract (0.5g) was stirred with 5mL of 1% HCl on a steam bath. The solution obtained was filtered and 1mL of the filtrate was treated with a few drops of Mayer's reagent. The turbidity of the extract filtrate on addition of Mayer's reagent was taken as evidence of the presence of alkaloids in the extracts.

### **2.5.3 Test for tannins and phenolics.**

Each extract (0.5g) was separately stirred with 10mL of distilled water and then filtered. Few drops of 5% FeCl<sub>3</sub> reagent was added to the filtrate. Blue-black, blue-green colouration and precipitation was taken as an indication of the presence of phenolics and tannins.

### **2.5.4 Test for phenols.**

Two (2) ml extract were taken into water and warmed at 50 °C. Then 2 ml of 3% FeCl<sub>3</sub> was added. Formation of green or blue color will indicate the presence of phenols.

### **2.5.5 Tests for steroids.**

- i. A red color produced in the lower chloroform layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid was added to it, indicates the presence of steroids.
- ii. Development of a greenish color when 2 ml of the organic extract was dissolved in 2 ml of chloroform and treated with sulphuric and acetic acid indicates the presence of steroids.

### **2.5.6 Keller-Killiani test for glycosides**

A total of 1 mL of glacial acetic acid, few drops of ferric chloride solution and conc. H<sub>2</sub>SO<sub>4</sub> (Slowly through the sides of the test tube) were added to the extract. Appearance of reddish brown ring at the junction of the liquids indicated the presence of de-oxysugars.

### **2.5.7 Test for Flavonoids**

Alkaline reagent test: Extract was treated with 10 % NaOH solution, formation of intense yellow colour indicated presence of Flavonoid

### **2.5.8 Test for phlobatannins**

One (1) ml extract was boiled with 2 ml of 1% hydrochloric acid. The red precipitate signified the presence of phlobatannins

### **2.5.9 Salkowski reaction test for Phytosterols**

To 0.5 mL chloroform extract in a test tube 1 mL of concentrated (conc.) H<sub>2</sub>SO<sub>4</sub> from the sides of the test tube was added. Appearance of reddish brown colour in chloroform layer indicates presence of phytosterols.

### **2.5.10 Test for Anthraquinones.**

Five (5) ml of the extract solution was hydrolysed with conc. H<sub>2</sub>SO<sub>4</sub> extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

### **2.5.11 Test for Coumarin**

Three (3) ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow colour indicates coumarins.

### **2.5.12 Test for Emodins**

Two (2) ml of NH<sub>4</sub>OH and 3 ml of benzene was added to extract appearance of red colour indicates presence of emodin.

### **2.5.13 Test for Amino acid/ Proteins Xanthoproteic**

Extract was treated with few drops of concentrated HNO<sub>3</sub>, formation of yellow indicated the presence of proteins.

## **2.6 Empirical scheme for the application of *Jatropha curcas* Root and Stem Extracts as anti-rabies vaccine in white mice**

### **2.6.1 Determination of lethal dose 50% (Ld<sub>50</sub>) of extraction**

The milligram dilutions of the extracts were prepared as follows in distilled water as follows:

#### **Preparation of Stock Solution**

The stock dilution of 250 mg was prepared by dissolving 2.5 g of the extract powder in 10 ml distilled water and allowed to stand for 1 hour before use. One milliliter of the stock dilution gives 1mg concentration of the extract.

2.5g of extract + 10ml distilled water = stock 1ml of stock = 250mg/ml

From the stock solution other mg concentration were prepared as follows

0.8ml of stock + 0.2ml distilled water = 200mg/ml

0.4ml of stock + 0.6ml distilled water = 100mg/ml

0.2ml of stock + 0.8ml distilled water = 50mg/ml

A 5000mg concentration was prepared as follows

Dissolve 5g of extraction powder in 5ml of water

This will give 5000mg stock

That is: 1ml of the 5000mg stock = 1000mg/ml

1ml of 5000mg stock + 2ml of water = 500mg/ml

### 2.6.2 Mice Inoculation

Seven cages with five mice containing in each of the seven cage of three weeks old was used to determination the lethal dose 50% (LD<sub>50</sub>) of the extract in milligram rate. Each mouse was given 0.5ml of the extract solution orally. The mice were allowed for three weeks with adequate feed and water. The animals were observed daily and any sick or dead mice was recorded the LD<sub>50</sub> was calculated using reed and muench (1938) simple estimation method.

### 2.6.3 LD<sub>50</sub> Calculation

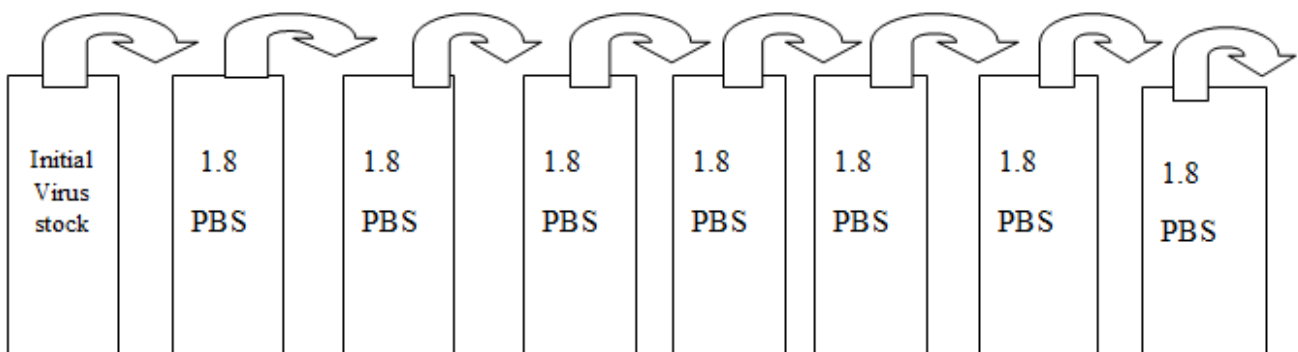
$$\text{Log LD}_{50} = \frac{\text{Net percentage above 50\%} - 50\%}{\text{Net percentage above 50\%} - \text{below 50\%}}$$

This will give the proportional distance from the actual dilution; hence it will be added to get the actual LD<sub>50</sub> mg concentration.

Determination of the rabies virus (challenge was standard – CVS) lethal dose 50(LD<sub>50</sub>)

The initial rabies stock from -20°C was allowed to thaw. An aliquot of 0.2ml of the CVS rabies virus was serially diluted in phosphate buffered saline (PHS), PH 7.2 and kept in ice trough. The dilution ranges from 10<sup>-1</sup> and 10<sup>-7</sup> and 0.03ml of each dilution was inoculated in to each mouse (0.03ml/mouse) per each dilution. The mice and the control mice were allowed for 14 days with feed and water.

### 2.6.4 Extract Dilution format



### 2.6.5 Virus (CVS) – Extract Reaction Test

Ten milliliters of the virus stock base on the LS<sub>50</sub> titration was prepared and kept ice pact. The different milligram concentration was prepared in bijou bottles. 0.3ml of each extract milligram of each concentration was mixed with 0.3ml of the virus stock and content was incubated at 4oc for 1 hour for the extract to react with the virus. 5 mice



were inoculated per milligram concentration was given to each 3 weeks old mice at the left or right thigh muscle or 0.3ml given through the brain (intra cerebrally). The inoculation and control mice were observed for 14 days. The sick or dead mice were recorded. The effectiveness or protection rates of the extracts were determined.

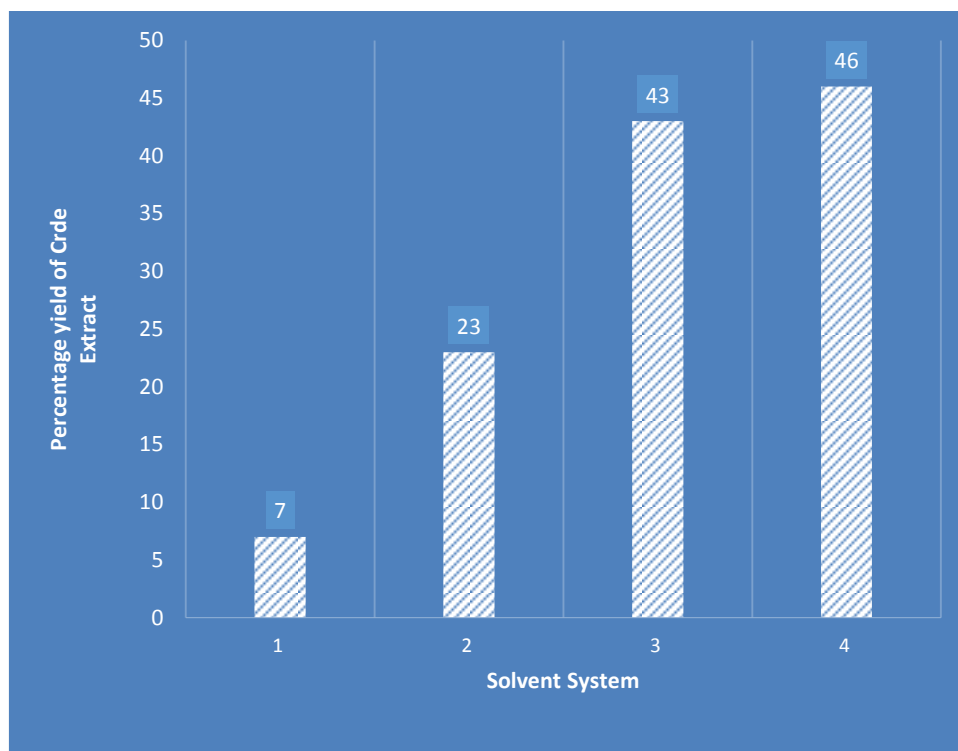
**Format:**

Extract sample	Extracts milligram concentrations				
EXTRACT 1	50mg	100mg	200mg	250m	500mg
EXTRACT 2	50mg	100mg	200mg	250mg	500mg
EXTRACT 3	50mg	100mg	200mg	250mg	500mg

**3.0 RESULT AND DISCUSSION**

**3.1 Results**

The tables titled 4.1 and 4.2 below shows the results gotten from the phytochemical screening of both root and stem bark extracts using four different suitable solvent systems which are hexane, benzene, ethanol and acetone. Individual procedure or method of phytochemical experiments were applied which produces the result of the tables bellow. Having a negative symbols against a phytochemical representing or shows the absence of a particular phytochemical component in the crude extract and the positive symbol representing the presence of the phytochemical in the crude extract that is analyzed.



**Fig. 4.1:** Histogram representing the Percentage yield of Plant Extract in Solvent Systems

In figure 4.1, the solvent systems 1, 2, 3, 4 represent hexane, ethanol, benzene and acetone solvent systems respectively. The figure suggests lowest yield in hexane while the highest yield is recorded in the benzene and

acetone solvent systems as supported by the results, giving more phytochemicals in the benzene and acetone systems than the hexane and ethanol systems. This indicate that benzene and acetone solvent systems are better choices for solvent extraction in plant sample matrix.

TABLE 4.1: Phytochemicals of root extract

Solvent system	PHYTOCHEMICALS												
	Steroid	Seponins	Phenols	Alkaloids	Glycosides	Coumarins	Tannins	Terpenoids	Anthocyanin	Amino acid	Plobatanins	Flavonoids	Emedins
Crude extract													
Hexane	-	-	-	-	+	-	-	+	-	+	-	-	-
Ethanol	+	+	+	+	-	-	-	+	+	+	-	+	+
Benzene	+	+	+	+	+	+	+	+	-	+	+	+	-
Acetone	+	+	+	+	+	+	-	+	+	+	+	+	+

Table 4.2: Phytochemicals of stem bark extract

Solvent system	PHYTOCHEMICALS												
	Steroid	Seponins	Phenols	Alkaloids	Glycosides	Coumarins	Tannins	Terpenoids	Anthocyanin	Amino acid	Plobatanins	Flavonoids	Emedins
Crude Extract													
Hexane	-	+	-	-	+	-	-	+	-	+	-	-	-
Ethanol	+	+	+	+	+	-	-	+	+	+	+	-	+
Benzene	+	+	+	+	+	+	+	+	-	+	+	+	-
Acetone	+	+	+	+	+	+	-	+	+	+	+	+	+

### 3.2 Discussion

The present study of phytochemical in tables one (1) and two (2) showed that between the stem bark and root extract analyzed, both are said to have more and effective phytochemically screened result considering the fact that they have similar result of acetone and benzene.

From table one (1) using acetone as a solvent system having that all the phytochemicals carried out on acetone solvent crude extract were found to be all positive (indicating presence of phytochemicals) with the absent of tennins. Another good solvent indicated in the research was benzene, consisting of eleven (11) positive phytochemicals out of thirteen (13) that were screened; only two were found to be absent (emodins and anthocyanin). Ethanol crude extract showed mixed results though much better then hexane, it (ethanol crude extract) was determined to have a positive of nine (9) and that of negative was four (4). Lastly hexane showed the lowest phytochemical screening result were the negative superseded the positive, having three (3) showing a positive screened result and ten (10) showing negative result.

On the second table stem back phytochemical screening similar to the result from the first table, acetone also shows a better result than any other in the table having only tannins to be negative and all other twelve (12) positive followed by benzene with eleven present and two absent. The stem back crude extract from ethanol shows ten (10) positive phytochemically screened result and three (3) negative lastly hexane consisting of two (2) positive result and eleven (11) negatives showing the most poorest result from all the phytochemically screened results from both tables.

Tables 1 and 2 presents the results of the phytochemical screening, which generally indicate for the presence of alkaloids, tannins, flavonoids, saponins, glycosides, steroids, phenols, flavonoid, coumarin, terpenoids, amino acid, phlobatannins, emodins and anthocyanin. The presence of these secondary metabolites in this research agrees with the work of other researchers (Ekundayo and Ekekwu, 2013; Prasad *et al*, 2012) and may contribute to the activity of the extracts against the test organisms. The present study does not only pave way for preliminary contribution to the medico-botany investing action but also shows a way for pharmacological research in future for the discovery of new sources of drugs from these phytochemicals (Sarmad *et al*, 2014; Nilofer *et al*, 2013; Prasad *et al*, 2012). The ethanolic extract in the root showed negative phytochemicals for glycosides, coumurins, tannins and plobatannins, whereas in the stem, it showed negative for coumurins, tannins, plobatannins and flavonoids. The result indicates deductively that the possible active phytochemicals in the plant for effective treatment of bacteriological infections could possibly be, tannins glycosides, coumurins, plobatannins and flavonoids (Ekundayo and Ekekwu, 2013).

The hydroxyl groups in phenol are thought to be responsible for the toxicity of the compounds to microorganisms. It inhibits enzymes through reaction with sulphhydryl groups or through nonspecific interaction with proteins/amino acid. Flavonoids have the ability to complex with extra cellular and soluble proteins and complex with bacterial cell walls (IPAN News, 2003). Phytochemical screening result showed that the antioxidant and antibacterial activities of the crude extracts of *J. curcas* depends on the presence of phytochemicals such as alkaloids, stenoids, flavonoids and tannis. This plants crude extracts could serve as potential material for combatting new antimicrobial and antioxidant agents as stipulated by other researchers (Ekundayo and Ekekwu, 2013; Prasad *et al*, 2012).

It has been established by researchers that flavonoids complex with the walls of the microbes, steroids to equilibrate the body conditions of animals, particularly mammals, whereas, phenols are antioxidants and indicative of poisoning capacity of the plant, which could be positive or negative (Hamid *et al*, 2011; Mariya *et al*, 2008; Wiliams *et al*, 2004; Kidd *et al*, 2001). Saponins natural tendency to ward off microbes makes them good candidates for treating fungal and yeast infections. These compounds served as natural antibiotics, which help the body to fight infections and microbial invasion (Erica *et al*, 2011). This study indicate the potentials of *J. Caucas* as a potential medicinal plant.

#### 4.0 CONCLUSION

The work shows that benzene and acetone are good solvent systems for the extraction of phytochemicals in plant samples, which is indicative of the availability of all screened phytochemicals in both extracts with the exception of tannins in benzene and anthocyanin in acetone.

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