QUALITATIVE PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF CRUDE EXTRACT FROM *Jatropha curcas* L., AGAINST SELECTED CLINICAL BACTERIAL ISOLATES

A PROJECT REPORT

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SANTHIYA J (0810204038)

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COIMBATORE

BONAFIDE CERTIFICATE

Certified that this project report **"Qualitative Phytochemical Analysis and antibacterial activity of crude extract from *Jatropha curcas* L., against selected clinical bacterial isolates"** is the bonafide work of **SANTHIYA. J. (0810204038)** who carried out the project work under my supervision.

SIGNATURE	**SIGNATURE**
SUPERVISOR	**HEAD OF THE DEPARTMENT**
Mr. M. Shanmugaprakash	**Dr. A. Manickam**
Assistant Professor (SrG)	Professor and Head
Department of Biotechnology	Department of Biotechnology
Kumaraguru College of Technology	Kumaraguru College of Technology
P. O. Box No. 2034	P. O. Box No. 2034
Chinnavedampatti	Chinnavedampatti
Coimbatore – 641 049	Coimbatore – 641 049
_____	_____
Internal Examiner	External Examiner

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[Santhiya. J]

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ABSTRACT

Jatropha curcas L. is an ornamental plant which is also employed to cure various infections in traditional medicine. In this study, fractions of the methanol, aqueous, ethyl acetate and hexane extracts from the *Jatropha curcas* latex and ethanolic extract from *Jatropha* leaves were tested for antibacterial properties against human pathogenic bacteria such as *Methicillin-resistant Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus, Escherichia coli, and Streptococcus mutans.* The zones of inhibition produced by the extracts using the disc diffusion method against the test microorganisms were found to range from 6 to 22 mm. The MIC values for the different extracts ranges from 10µg/ml - 640 µg /ml . Leaf and aqueous extracts did not show any antibacterial activity against *K.pneumoniae***.** Phytochemical analysis of the extract and latex revealed the presence of many secondary metabolites including tannins, alkaloids and saponins. TLC separation also revealed the presence of phenolic acid in the plant extracts.

KEY WORDS: *Jatropha curcas L., antibacterial activity, phytochemicals, Secondary metabolites.*

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LIST OF ABBREVIATIONS

H_2O (L)	Aqueous extract of latex
EC	*E.coli*
EtOAc (L)	Ethylacetate extract of latex
EtOAc P (L)	Ethylacetate extract of latex protected by Sodium benzoate
FFA	Free Fatty Acid
Hex (L)	Hexane extract of latex
K	*K.pneumoniae*
L	Latex
MeOH (L)	Methanol extract of latex
-	Negative
++	Positive
P	*Proteus*
PA	*Pseudomonas aeruginosa*
+	Slightly positive
SA	*Staphylococcus aureas*
SM	*Streptococcus mutans*
T^{10}	Tetracyclin

CHAPTER 1

INTRODUCTION

Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. Recent estimations suggest that over 9,000 plants have been known for medicinal applications in various cultures and countries, and this is without having conducted comprehensive research amongst several indigenous and other communities (Jain, 1996). The search for plants with antimicrobial activity has gained increasing importance in recent years, due to a growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistant microorganisms or multi-resistant microbes.

The extensive use of antibiotics has led to the rapid emergence of multi-drug resistance among pathogenic bacteria; for example, penicillin which was introduced in the 1930's was initially hyped to be the "magic bullet" against pathogenic bacteria is now much less effective as bacterial strains develop resistance against the antibiotic (Shamweel and Dar, 2011). The pharmaceutical industry is struggling to keep up with the production of new synthetic antibiotics as more and more bacterial strains develop resistance against existing drugs. The con-sumption of synthetic antimicrobials are known to affect the human body because of the disruption to the natural human microbiota which are known to play key roles in nutrition, development, metabolism, pathogen resistance and regulation of the human immune responses (Dethlefsen *et al*., 2008). There are many side effects of synthetic antibiotics towards the human body such as diarrhea which is caused by the disruption of the normal intestinal floral which induces the growth of *Clostridium difficile* (anaerobic bacteria) (Pirotta and Garland, 2006). Therefore, there is a pressing need for the development of new antimicrobial drugs particularly from natural sources as these may reduce the risk of toxicity observed in synthetic antibiotic.

Also, before an antimicrobial agent is accepted for use in human beings it must demonstrate most, if not all, of the following properties: selective toxicity (it should act on bacteria without damaging the host tissues); it should be bactericidal rather than bacteriostatic; it

should be effective against a broad range of bacteria; it should not be allergic; it should remain active in plasma, body fluids etc.; it should be stable and preferably water soluble; desired levels should be reached rapidly and maintained for adequate period of time; it should not give rise to resistance in bacteria; it should have long shelf life; it should not be expensive (Rajesh and Rattan, 2008).

The effectiveness of chemotherapeutic agents depend on many factors, some of which include; the route of administration and location of the infection, the presence of interfering substances, the concentration of the drug in the body, the nature of the pathogen, the presence of drug allergies, and another factor that should not be overlooked is the resistance of microorganisms to the drug. The increasing number and the variety of drug resistance pathogen is a serious public health problem. Bacteria often become resistant in several different ways. Unfortunately, a particular type of resistance mechanism is not confined to a single class of drugs. Two bacteria may use different resistance mechanisms to withstand the same chemotherapeutic agent. Furthermore, resistant mutants arise spontaneously and are then selected for in the presence of the drug. Bacteria can become resistant to a drug by excluding it from cell, pumping the drug out of the cell, enzymatically altering it, modifying the target enzymes or organelles to make it less drug sensitive (Willey *et al*., 2008).

Plants are widely used for medicinal purpose in different countries and are a source of many potent and powerful drugs (Srivastava *et al.,* 1996). Parts of *Jatropha curcas* have been used in traditional medicine and for veterinary purposes in regions where the plant is grown (Gübitz *et al.,* 1999). The latex is used to treat fungal infections in the mouth, bee and wasp stings and digestive problems of children in Mexico (Schmook and Serralta-Peraza, 1997). Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new, antimicrobial compounds (Dygerak *et al.,* 2002; Ibrahim *et al*., 2002; Bassam *et al.,* 2006). The interest in the scientific investigation of *Jatropha curcas* L. is based on the claims of its effective use for the treatment of many diseases.

The sap (latex) also has antimicrobial properties against *Staphylococcus* and *Streptococcus sp.*, and *E. coli*. Latex is used to dress sores, ulcers and inflamed tongues. Latex

from the stem is used to arrest bleeding of wounds. Seeds are used for dropsy, gout, paralysis and skin ailments (Watt and Breyer-Brandwijk, 1962). Therefore, this research regarding the antimicrobial activity of this plant is expected to enhance the use of *Jatropha* against diseases caused by the test pathogens. It is expected that screening of plant extract against wide variety of test organisms will be helpful in obtaining new antimicrobial substances. The action of the plant on microorganisms have been found to be due to the presence of certain substances such as alkaloids, glycosides, tannins, steroids, saponins, flavonoids and other chemical compounds referred to as secondary metabolites that are present in them.

1.1. OBJECTIVES

- Extraction of leaves and latex of *Jatropha curcas* L., using solvents like Ethanol, Ethyl acetate, Methanol, Water and Hexane.
- Determination of the antimicrobial activity of crude leaf extract and various fractions of crude latex of *J. curcas* against six selected human pathogenic bacteria.
- Qualitative analysis of the phytochemicals present in *Jatropha curcas.*
- Determination of Minimum Inhibitory Concentration.
- Separation of phytochemicals of different extracts using Thin Layer Chromatography (TLC).

CHAPTER 2
LITERATURE REVIEW

Jatropha, a drought-resistant shrub or tree, which is widely distributed in the wild or semi-cultivated areas in Central and South America, Africa, India and South East Asia (Cano-Asseleih *et al.,* 1989). The first commercial applications of *Jatropha* were reported from Lisbon, where the oil imported from Cape Verde was used for soap production and for lamps. In addition to being a source of oil, *Jatropha* also provides a meal that serves as a highly nutritious and economic protein supplement in animal feed, if the toxins are removed (Becker and Makkar, 1998). The plant can be used to prevent soil erosion, to reclaim land, grown as a live fence, especially to exclude farm animals and also planted as a commercial crop (Heller, 1996). Various parts of the plant are of medicinal value, its bark contains tannin, the flowers attract bees and thus the plant has a honey production potential. Its wood and fruit can be used for numerous purposes including fuel. It is easy to establish and grows relatively quickly.

2.1. TAXONOMY AND BOTANICAL DESCRIPTION:

- Kingdom: Plantae
- Sub Kingdom: Tracheobionta
- Division: Magnoliophyta
- Class: Magnoliopsida
- Subclass: Rosidae
- Order: Euphorbiales
- Family: Euphorbiaceae
- Genus: *Jatropha*
- Species: *curcas*

The genus *Jatropha* belongs to tribe Joannesieae in the Euphorbiaceae family and contains approximately 170 known species. Linnaeus (1753) was the first to name the physic nut *Jatropha* L. in "Species Plantarum" and this is still valid today.

The genus name *Jatropha* derives from the Greek word *jatr´os* (doctor) and *troph´e* (food), which implies medicinal uses. The physic nut, by definition, is a small tree or large shrub, which can reach a height of three to five meters, but under favourable conditions it can attain a

height of 8 or 10m. The plant shows articulated growth, with a morphological discontinuity at each increment. The branches contain latex. Normally, five roots are formed from seedlings, one central and four peripheral. A tap root is not usually formed by vegetatively propagated plants. Leaves five to seven lobed, hypostomatic and stomata are of paracytic (Rubiaceous) type.

The trees are deciduous, shedding the leaves in dry season. Flowering occurs during the wet season and two flowering peaks are often seen, i.e. during summer and autumn. In permanently humid regions, flowering occurs throughout the year. The inflorescence is axillary paniculate polychasial cymes. The plant is monoecious and flowers are unisexual; occasionally hermaphrodite flowers occur (Dehgan and Webster, 1979). A flower is formed terminally, individually, with female flowers (tricarpellary, syncarpous with trilocular ovary) usually slightly larger and occurs in the hot seasons.

In conditions where continuous growth occurs, an unbalance of pistillate or staminate flower production results in a higher number of female flowers. Ten stamens are arranged in two distinct whorls of five each in a single column in the androecium, and in close proximity to each other. In the gynoecium, the three slender styles are connate to about two-thirds of their length, dilating to massive bifurcate stigma (Dehgan and Webster, 1979). The rare hermaphrodite flowers can be self pollinating.

The flowers are pollinated by insects especially honey bees. Each inflorescence yields a bunch of approximately 10 or more ovoid fruits. With good rainfall conditions nursery plants may bear fruits after the first rainy season, and directly sown plants after the second rainy season. Three, bivalved cocci is formed after the seeds mature and the fleshy exocarp dries**.** The seeds mature about 3–4 months after flowering.

The seeds are black and the seed weight per 1000 is about 727 g, there are 1375 seeds/kg in the average. (Singh, 1970) described the microscopical anatomy of fruits. (Gupta, 1985) investigated the anatomy of other plant parts. The physic nut is a diploid species with $2n = 22$ chromosomes.

Table 2.1. Various species of *jatropha*

Jatropha curcas	*Jatropha gossypifolia*	*Jatropha glandulifera*
Jatropha heynei	*Jatropha integerrima*	*Jatropha maheshwarii*
Jatropha multifida	*Jatropha mulendnifera*	*Jatropha villosa*
Jatropha nana	*Jatropha podagrica*	*Jatropha hastata*
Jatropha tanjovurensis	*Jatropha hastata*	*Jatropha macrofayala*
Jatropha acrocurcas	*Jatropha diyoka*	*Jatropha sinera*

Out of above, *Jatropha curcas* gained prominence because of its added features like excellent adaptability to various habitats, larger fruits and seeds, high oil yielding, soil conservation capabilities, thriving well as live fence etc.

Table 2.2. Chemicals isolated from different parts of the plant:

Various Parts	Chemical composition	References
Aerial parts	Organic acids (*o* and *p*-coumaric acid, *p*-OH-benzoic acid, protocatechuic acid, resorsilic acid, saponins and tannins	Hemalatha and Radhakrishnaiah (1993)
Stembark	β-Amyrin, β-sitosterol and taraxerol	Mitra *et al*. (1970)
Leaves	Cyclic triterpenes stigmasterol, stigmast-5-en-3β, 7β-diol, stigmast-5-en-3β,7α-diol, cholest-5-en-3β,7β-diol, cholest-5-en-3fl,7α-diol, campesterol, β-sitosterol, 7-keto- β -sitosterol as well as the β-D-glucoside of β -sitosterol. Flavonoids apigenin, vitexin, isovitexin	Mitra *et al*. (1970), Khafagy *et al*. (1977), Hufford and Oguntimein (1987)
	Leaves also contain the dimer of a triterpene alcohol ($C_{63}H_{117}O_9$) and two flavonoidal glycosides	Khafagy *et al*. (1977)
Latex	Curcacycline A, a cyclic octapeptide	Van den Berg *et al*. (1995)
	Curcain (a protease)	Nath and Dutta (1991)
Seeds	Curcin, a lectin	Stirpe *et al*. (1976)
	Phorbolesters	Adolf *et al*. (1984), Makkar *et al*. (1997)
	Esterases (JEA) and Lipase (JEB)	Staubmann *et al*. (1999)
Kernal and press cake	Phytates, saponins and a trypsine inhibitor	Aregheore *et al*. (1997), Makkar and Becker (1997), Wink *et al*. (1997)
Roots	β-Sitosterol and its β-D-glucoside, marmesin, propacin, the curculathyranes A and B and the curcusones A–D. diterpenoids jatrophol and jatropholone A and B, the coumarin tomentin, the coumarino-lignan jatrophin as well as taraxerol.	Naengchomnong *et al*. (1986, 1994)

2.2. SEED AND ITS TOXICITY

The seeds of physic nut are a good source of oil, which can be used as a diesel substitute. However, the seeds of *J. curcas* are, in general, toxic to humans and animals. Curcin, a toxic protein isolated from the seeds, was found to inhibit protein synthesis in in vitro studies. The high concentration of phorbol esters present in Jatropha seed has been identified as the main toxic agent responsible for Jatropha toxicity (Adolf *et al.,* 1984; Makkar *et al.,* 1997). These phorbol esters are found in plants belonging to the families Euphorbiaceae and Thymelaeaceae (Ito *et al.,* 1983). Several cases of *J. curcas* nut poisoning in humans after accidental consumption of the seeds have been reported with symptoms of giddiness, vomiting and diarrhoea and in the extreme condition even death has been recorded (Becker and Makkar, 1998). Ionizing radiation treatment could serve as a possible additional processing method for

inactivation or removal of certain anti-nutritional factors such as phorbol esters, phytates, saponins and lectins (Siddhuraju *et al.,* 2002). It is not possible to destroy phorbol esters by heat treatment because they are heat stable and can withstand roasting temperature as high as 160 ˚C for 30 min.

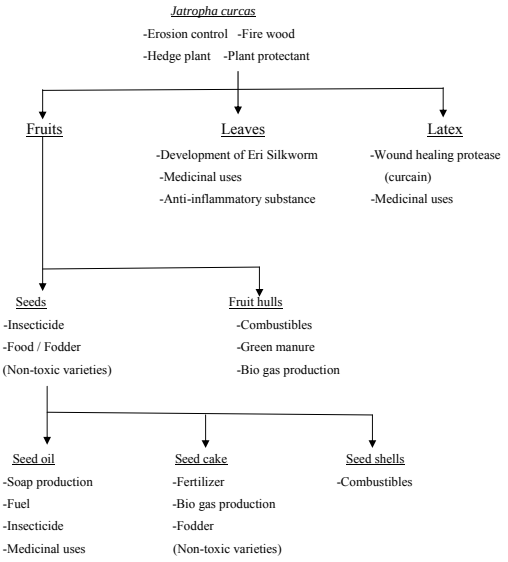


Fig 2.1. Exploitation of *Jatropha* (Gubitz *et al.,* 1999)

However, it is possible to reduce its concentration in the meal by chemical treatments. This treatment is promising, but in economic terms it is expensive to produce *Jatropha* meal from it (Aregheore *et al.,* 2003). (Mart´ınez-Herrera *et al.*, 2006) studied the nutritional quality and the effect of various treatments (hydrothermal processing techniques, solvent extraction, solvent extraction plus treatment with $NaHCO_3$ and ionizing radiation) to inactivate the antinutritional factors in defatted *Jatropha* kernel meal of both toxic and non-toxic varieties from different regions of Mexico. Complete removal of the toxins is therefore necessary before *Jatropha* oil can be used in industrial applications or in human medicine, the oilmust be shown to be completely innocuous before it is used commercially.

2.3. USES OF *JATROPHA* PRODUCTS

2.3.1. As hedge

Jatropha is an excellent hedging plant generally grown in most part of India as live fence for protection of agricultural fields against damage by livestock as unpalatable to cattle and goats. Thus in addition to seed yields it serves the purpose of bio fence with respect to cost effectiveness as compared to wire fence.

2.3.2. As green manure and fertilizers

Seed cake or press cake is a by-product of oil extraction. *Jatropha* seed cake contains curcin, a highly toxic protein similar to ricin in castor, making it unsuitable for animal feed. However, it does have potential as a fertilizer or biogas production (Staubmann *et al.,* 1997; Gubitz *et al.,* 1999), if available in large quantities; it can also be used as a fuel for steam turbines to generate electricity. The defatted meal has been found to contain a high amount of protein in the range of 50–62%, and the level of essential amino acids except lysine is higher than the FAO reference protein (Makkar *et al.,* 1998).

Being rich in nitrogen, the seed cake is an excellent source of plant nutrients. In a green manure trial with rice in Nepal, the application of 10 tonnes of fresh physic nut biomass resulted in increase yield of many crops (Sherchan *et al.,* 1989). Another use of *Jatropha* seed cake is as a straight fertilizer, its properties were compared with those of other organic fertilizers with regard to nitrogen, phosphorus and potassium content which is shown in the Table 2.3.

Table 2.3. Nutritional analysis of oil seed cakes and manure % (Delgado and parado, 1989)

Property	*J. curcas* oil cake	Neem oilcake	cow manure
Nitrogen	3.2-4.44	5.0	0.97
Phosphorus	1.4-2.09	1.0	0.69
Potassium	1.2-1.68	1.5	1.66

In preliminary experiment, *Jatropha* seed cake is utilized as feedstock for biogas production (Karve, 2005; Visser and Adriaans, 2007). Experiments on use of biogas slurry as a fertilizer are still in the early stages. Recently experimentation on solid-state fermentation of *Jatropha* seed cake showed that, it could be a good source of low cost production of industrial enzymes (Mahanta *et al.,* 2008).

2.3.3. As food

The physic nut seed is eaten in certain regions of Mexico once it has been boiled and roasted (Delgado and Parado, 1989). *Jatropha* can be toxic when consumed, however, a non-toxic variety of *Jatropha* is reported to exist in some provenances of Mexico and Central America, said not to contain toxic Phorbol esters (Makkar *et al.,* 1998). This variety is used for human consumption after roasting the seeds/nuts, and "the young leaves may be safely eaten, steamed or stewed" (Duke, 1985a; Ochse, 1931). (Sujatha *et al.,* 2005) have been established the protocols for *in vitro* propagation of non-toxic variety of *Jatropha* through axillary bud proliferation and direct adventitious shoot bud regeneration from leaf segments.

2.3.4. As soap

The glycerin that is a by-product of biodiesel can be used to make soap, and soap can be produced from *Jatropha* oil itself. In either case the process produces a soft, durable soap and is a simple one, well adapted to household or small-scale industrial activity.

2.3.5. As Pesticide

The oil and aqueous extract from oil has potential as an insecticide. For instance it has been used in the control of insect pests of cotton including cotton bollworm and on pests of pulses, potato and corn (Kaushik and Kumar, 2004). Methanol extracts of *Jatropha* seed (which contains biodegradable toxins) are being tested in Germany for control of bilharzias-carrying water snails.

2.3.6. As Charcoal

In simple charcoal making, 70–80% of wood energy is lost with yield of only 30% in an industrial process, where charcoal is still one of the few simple fuel options. *Jatropha* wood is a very light wood and is not popular as a fuel wood source because it burns too rapidly. The use of press cake as a fertilizer is more valuable to increase crop production then charcoal making from it (Benge, 2006). However, the extraction of oil from *Jatropha* seeds is of much higher economic value than converting the wood to charcoal. Converting *Jatropha* seed shells into charcoal would be economically feasible, only if we have a large source of seed shells from *Jatropha* plantations. The scientist concluded that *Jatropha* wood would not be of much value for either charcoal or firewood (Benge, 2006).

2.3.7. Medicinal uses

All parts of *Jatropha* (seeds, leaves and bark) have been used in traditional medicine and for veterinary purposes for a long time (Dalziel, 1955; Duke, 1985b; Duke, 1988). Some compounds (Curcacycline A) with antitumor activities were reportedly found in this plant (Van den Berg *et al.,* 1995). Substances such as phorbol esters, which are toxic to animals and humans, have been isolated and their molluscicidal, insecticidal and fungicidal properties have been demonstrated in lab-scale experiments and field trials (Nwosu and Okafor, 1995; Solsoloy and Solsoloy, 1997).

The seed oil can be applied to treat eczema and skin diseases and to soothe rheumatic pain (Heller, 1996). The 36% linoleic acid (C18:2) content in *Jatropha* kernel oil is of possible interest for skincare. Furthermore, (Goonasekera *et al.,* 1995) showed that various solvent extracts of *Jatropha* have an abortive effect. The oil has a strong purgative action and is also widely used for skin diseases and to soothe pain such as that caused by rheumatism. The oil is used as a cathartic purgative (Jamalgota) and for the treatment of skin ailments (Duke, 1988).

The latex itself has been found to be strong inhibitors to watermelon mosaic virus (Tewari and Shukla, 1982). The leaves and latex are used in healing of wounds, refractory ulcers, and septic gums and as a styptic in cuts and bruises. A proteolytic enzyme (curcain) has been reported to have wound healing activity in mice (Nath and Dutta, 1997; Villegas *et al.,* 1997). Investigation of the coagulant activity of the latex of *Jatropha* showed that whole latex significantly reduced the clotting time of human blood. Diluted latex, however, prolonged the clotting time, at high dilutions, the blood did not clot at all (Osoniyi and Onajobi, 2003). Topical application of *Jatropha* root powder in paste form is common ethnobotanical practices for the treatment of inflammation, which has been followed by Bhil tribes from Rajasthan area in India and it was confirmed in albino mice and the successive solvent extraction of these roots was carried out by ether and methanol. The methanol extract of these roots exhibited systemic and significant anti-inflammatory activity in acute carrageenan-induced rat paw edema (Mujumdar and Misar, 2004). Economic significance of Jatropha are presented in Fig. 2.2.

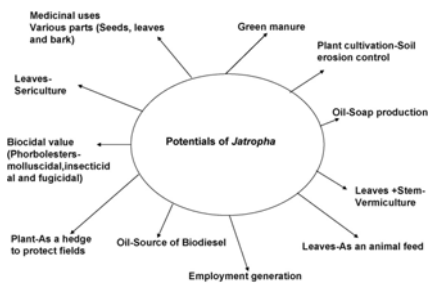


Fig 2.2. Economic significance of *J. curcas*

2.4. AS AN ENERGY SOURCE

The oil from *Jatropha* is regarded as a potential fuel substitute. The types of fuels, which can be obtained directly from the *Jatropha* plant, are; wood, the whole fruit and parts of the fruit which can be burnt separately or in combination. Processing increases the energy value of the product, but the overall energy availability decreases unless a use can be found for the by-products. Recently novel approach is developed for extraction of oil from seed kernel of *Jatropha* by using enzyme assisted three-phase partitioning (Shah *et al*., 2004).

2.4.1.Biodiesel from physic nut

Biodiesel is made from virgin or used vegetable oils (both edible and non-edible) and animal fats through transesterification and is a diesel substitute and requires very little or no engine modifications up to 20% blend and minor modification for higher percentage blends. *Jatropha* oil can be used as fuel in diesel engines directly and by blending it with methanol (Gubitz *et al*., 1999). The seed oil of *Jatropha* was used as a diesel fuel substitute during the World War II. Engine tests with *Jatropha* oil were done in Thailand, showing satisfactory engine performance (Takeda, 1982). For African countries, the feasibility of the production of fatty acid ethyl esters from *Jatropha* oil was studied (Eisa, 1997). The economic evaluation has shown that the biodiesel production from *Jatropha* is very profitable provided the by-products of the biodiesel production can be sold as valuable products (Foidl and Eder, 1997). Berchmans and Hirata (2008) and Tiwari *et al*. (2007) have been developed a technique to produce biodiesel from *Jatropha* with high free fatty acids contents (15% FFA), in which two-stage transesterification process was selected to improve methyl ester yield. The first stage involved the acid pretreatment process to reduce the FFA level of crude *Jatropha* seed oil to less than 1% and second was the alkali base catalyzed trans-esterification process gave 90% methyl ester yield. In order to reduce the cost of biodiesel fuel production from *Jatropha*, the lipase producing whole cells of *Rhizopus oryzae* immobilized onto biomass support particles was used and found to be a promising biocatalyst for producing biodiesel (Tamalampudi *et al*., 2007).

More efficient expeller system can be used to extract a higher % of oil from the seeds, which in turn should produce higher profits in a *Jatropha* system, since oil sells for more than the residual seed cake. The simple technology specially developed for this chemical process can also be performed in less industrialized countries (Mittelbach *et al*., 1983; Connemann, 1994). Use of methyl ester of *Jatropha* oil and dual fuel operation with methanol induction can give better performance and reduced smoke emissions than the blend. Dual fuel operation showed the lowest smoke and NO levels (Senthil *et al.,* 2003). Sarin *et al*., (2007) have examined the blends of *Jatropha* and Palm biodiesel for their physico-chemical properties and to get optimum mix of them to achieve better low temperature and improved oxidation stability needed for South Asian and South East-Asian countries.

2.5. NON-ENERGY SOURCE

It is a woody plant and, therefore, its various parts can be used for a number of purposes, especially as fuel, sticks and poles. In some countries, the live pole is used to support vines such as the vanillin plant. Bees pollinate their flowers, thus it is possible to have apiaries in association with *Jatropha* areas. A varnish can be made from the oil and the leaves could be feedstock for silk worms but not everywhere. For example, the experiments conducted by the authors, on rearing of *Philosamia ricini* on *Jatropha* leaves, concluded 100% mortality of erisilk worm *P. ricini* on leaf biomass.

(Ramappa raghavendra and gurumurthy, 2011) have determined antimicrobial activity of latex of various medicinal plants like *Carica papaya, Calatropes procera, Artocarpus heterophyllus, Jatropha carcass and Thevetia peruviana* against certain human pathogenic micro organisms.

(Akpan and Ojo, 2008) have reported antimicrobial activity of *Calatropes procera* plant latex against six microbes.

CHAPTER 3
MATERIALS AND METHODS

3.1. COLLECTION OF PLANT MATERIALS

Jatropha curcas latex & leaf samples were collected from medicinal garden, Kumaraguru College of Technology, Coimbatore, Tamilnadu. The stem/branch of the tree was cut and a bottle was hanged to collect the fresh latex. The plants used for latex collection were two years old, having been planted in 2010. Latex was collected into vials containing a few drops of 95% ethanol in one set and sodium benzoate in other set to prevent browning and oxidation. Then it is stored at -4˚C until further usage.

3.2. BACTERIAL STRAINS

Six human pathogenic bacteria, namely *Streptococcus mutans* (MTCC 497), *E. coli, Klebsiella pneumoniae, Proteus, Staphylococcus aureas, Pseudomonas aeruginosa* were used in this study to test for antimicrobial properties of the *J. curcas* plant extracts . *E. coli, Klebsiella pneumoniae, Proteus, Staphylococcus aureas, Pseudomonas aeruginosa* were collected from Immuno lab, RS Puram, Coimbatore. The bacteria were selected based on their pathogenicity onto human body. The bacteria were cultured in nutrient agar, NA (Difco) to obtain pure single colony by appropriate four quadrants streaking on the solid media and incubating the agar plates overnight at 37°C.

3.3. PREPARATION OF PLANT EXTRACTS

A known quantity of the fresh latex (50 ml) was successively extracted with 100ml each of hexane, ethyl acetate, methanol and water by keeping it in the orbital shaker for two days overnight at room temperature.

Fresh leaves of *Jatropha* were collected from a 2 year old shrub growing in Kumaraguru college of technology, Coimbatore, India and used for experimental work. Leaves were dried at room temperature and then ground into fine powder using a grinder. A sample (20 gm.) of powdered plant material was exhaustively extracted by soxhlet extraction method using 80% Ethanol. At the end of the extraction, extract was filtered using Whatman filter paper. The filtrate was concentrated in vacuum at 30°C and stored at 4°C until further use. The final crude extract was diluted with ethanol to a standard volume and tested separately against the test organisms.

3.4. STERILITY TEST OF THE LEAF EXTRACT AND LATEX

Each of the leaf and latex extracts were tested for growth or contaminants. This was carried out by inoculating 1ml of each of them on nutrient agar and incubated at 37^{o}C for 24 hours. The plates were observed for growth. No growth in the extracts and latex after incubation indicated that the extracts and latex were sterile. The extracts and latex were then assessed for antimicrobial activity.

3.5. DETERMINATION OF ANTIMICROBIAL ACTIVITY

The filter paper disc method is used for screening of crude extract for antimicrobial activity. Standard size blank Whatman filter paper discs, 5.00 mm. in diameter, sterilized by dry heat at 140°C for 1 hour, were saturated with the extract (2 ml) and known quantity of standard reference antibiotics separately. Now the discs were air dried at room temperature to remove any residual solvent which might interfere with the determination. The discs were then placed on the surface of the sterilized Mueller-Hinton agar medium that had been inoculated with the test organism by using a sterile swab and air dried to remove moisture.

The thickness of the agar medium was kept equal in all Petri plates and the standard discs of Tetracyclin were used, separately for all microbes tested, in the petriplates as control. Before incubations, petri plates were placed for 1 hour in a cold room (4 °C) to allow diffusion of the compounds from the disc into agar plate. These were incubated at 37 °C for 20 to 24 hours, after which the zones of inhibition of desired growth could be easily measured. The zone of inhibition was considered as an indicator for the antimicrobial activity. At the end of the incubation period the antibacterial activity was evaluated by measuring the inhibition zones.

3.5. MINIMUM INHIBITORY CONCENTRATION (MIC) DETERMINATION

The test was performed in 12 well plates using two-fold dilutions of the crude extracts. A 100 µl aliquot of bacterial culture was added to each well. This was followed by 100 µl of the crude extracts. With each subsequent well, the concentration of the crude extract was diluted. The 12 well plates were then kept at 37°C overnight. To determine the viability of the bacterial cultures in the wells, a colorimetric assay was utilized whereby 20 µl MTT diluted in 20% DMSO (it was chosen because it has no effect to the bacterial growth) was added to each well and left to incubate at 37°C for 20 min. The appearance of a yellow colour indicates that the bacterial culture is not viable and has been killed by the presence of the crude extract. Viable cultures will appear blue in color.

3.6. PHYTOCHEMICAL SCREENING OF THE EXTRACTS OF LEAF AND LATEX OF *Jatropha curcas* L.

The methods described by (Odebiyi and Sofowora 1978) were used to test for the presence of saponins, tannins, phenolics and alkaloids, while the Salkowski test was used to test for the presence of glycosides.

Testing for saponins: Each extract (0.5g) was mixed with water in test tube. Foaming which persisted on warming was taken as an evidence for the presence of saponins.

Testing for tannins and phenolics: Each extract (0.5g) was separately stirred with 10ml of distilled water and then filtered. Few drops of 5% $FeCl_3$ reagent was added to the filtrate. Blue-black or blue-green colouration or precipitation was taken as an indication of the presence of phenolics and tannins.

Testing for alkaloids: Each extract (0.5g) was stirred with 5ml of marquis reagent. Orange-brown coloration was taken as evidence of the presence of alkaloids in the extracts.

Testing for flavonoids: 0.5g of each extract was separately added with few drops of 1% aluminium solution. The appearance of a yellow colour indicated the presence of flavonoids.

Testing for glycosides: 0.5g of each extract was dissolved in 2ml of chloroform. Tetraoxosulphate VI acid (H_2SO_4) was carefully added to form a lower layer. A reddish brown colour at the interface indicated the presence of a steroidal ring, that is, a glycone portion of the cardiac glycosides.

3.8. THIN LAYER CHROMATOGRAPHIC (TLC) SEPARATION OF PLANT EXTRACTS

Silica gel is uniformly coated on glass plate and plant extracts are spotted on the stationary phase. 200ml of Chloroform: Methanol: water in the ratio of 5:4:1 is saturated in a glass chamber. After 24 hours spotted plates in made to run using the mobile phase. It is sprayed with liquid ammonia for the identification of Phenolic acid which shows blue color spots under long UV transillumination confirming the presence of Phenolic acid in the plant extracts.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. ANTIBACTERIAL ANALYSIS

Leaf and Hexane extracts of latex showed maximum sensitivity towards *S.mutans.* Ethylacetate extract showed maximum sensitivity towards *P.aeruginosa, Staphylococcus aureas and E.coli.* Hexane extract showed maximum sensitivity towards *Proteus and Klebsiella pneumonia.*

Table 4.1. Antibacterial analysis of different extracts

EXTRACTS (0.64 mg/ml)	**Microorganisms / zone of inhibition (mm)**					
	SM	**EC**	PA	K	P	SA
Leaf	22	8	7	__	6	10
H_2O (L)	14	11	6	__	8	9
MeOH (L)	11	11	8	6	8	10
EtOAc (L)	10	19	10	8	11	13
EtOAc P (L)	15	11	7	7	8	7
Hex (L)	17	13	9	11	12	12
T^{10}	30	22	8	22	34	30

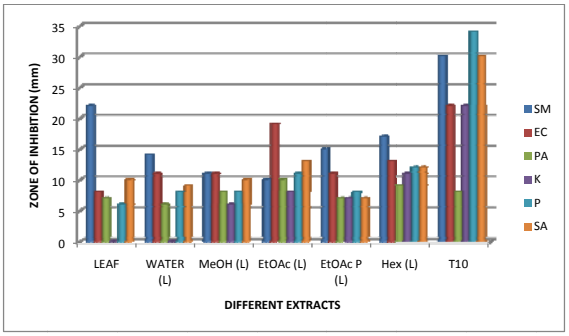


Fig 4.1. Overall diameter of inhibitory zone of different *J. curcas* fractions against various types of bacteria

Fig 4.4. Antibacterial activity of various extracts against *K. pneumoniae* (0.64 mg/ml)

Fig 4.5. Antibacterial activity of various extracts against *Proteus* (0.64 mg/ml)

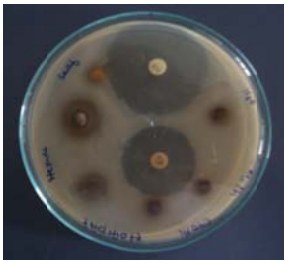


Fig 4.2. Antibacterial activity of various extracts against *Streptococcus mutans* (0.64 mg/ml)

Fig 4.3. Antibacterial activity of various extracts against *E. coli*(0.64 mg/ml)

Fig 4.6. Antibacterial activity of various extracts against *Staphylococcus aureus* (0.64 mg/ml)

Fig 4.7. Antibacterial activity of various extracts against *Pseudomonas aeruginosa* (0.64 mg/ml)

4.2. QUALITATIVE PHYTOCHEMICAL ANALYSIS

From the qualitative analysis it is found that leaf extract lack Saponins and Glycosides. Also latex extracts are rich in saponins, tannins, phenolics, alkaloids, flavonoids and glycosides.

Since latex is rich in secondary metabolites like saponins and tannins possess more anti-microbial activity compared to leaf extract.

Since both latex and leaf extracts are rich in Flavonoids and phenolics, it also possess good anti-oxidant activity.

Table 4.2. Qualitative phytochemical analysis

Extracts	Saponins	Tannins& phenolics	Alkaloids	Flavonoids	Glycosides
Leaf	-	++	++	+	-
H2O (L)	++	++	++	+	++
EtOAc (L)	++	++	++	++	+
EtOAc P (L)	++	++	++	++	++
MeOH (L)	++	++	++	+	+
Hex (L)	++	+	++	+	++

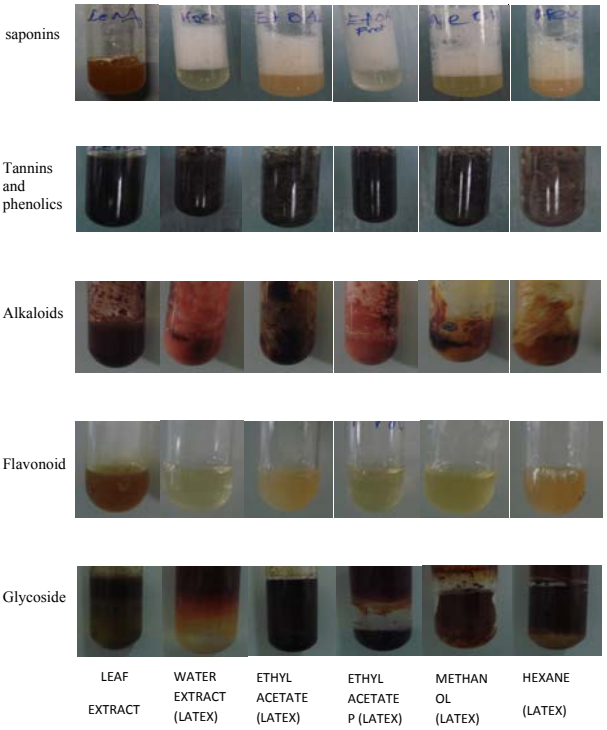


Fig 4.8. Qualitative phytochemical analysis

4.3. Minimum Inhibitory Concentration (MIC) Assay:

The MIC assay was carried out to determine the lowest concentration of the crude extract that would be inhibitory to the growth of the bacterial strain (Table 4.3.). This was performed starting with the lowest concentration of the crude extract that was inhibitory to the bacterial growth and was successively diluted 2-fold. The MIC value was taken at the lowest concentration in which inhibition to bacterial growth was observed. Minimum inhibitory concentration ranges from 10µg/ml – 640 µg/ml.

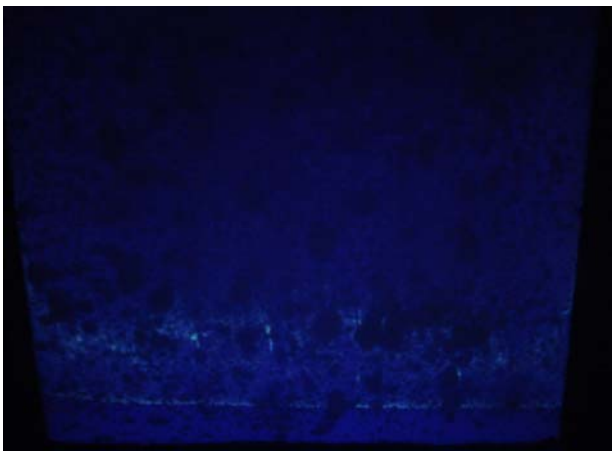
For SM, P& SA, MIC is 10 µg/ml; For K & EC, MIC is 20 µg/ml; For PA, MIC is 80 µg/ml.

Table 4.3. Minimum Inhibitory Concentration (MIC) for test organisms

EXTRACTS	K	EC	SA	SM	PA	P
LEAF	-	160	40	10	160	160
H2O (L)	-	160	320	10	320	160
EtOAc (L)	640	20	10	80	80	40
EtOAc P(L)	320	160	80	160	640	160
MeOH	40	160	40	80	160	320
Hex	20	80	20	20	320	10

4.4. TLC SEPARATION:

Separated extracts after spraying ammonia solution turned blue color representing the presence of Phenolic acids. They have very good anti-oxidant properties. Hence the protect against oxidative damage diseases like Coronary heart disease, stroke and cancers. The separation is shown in figure 4.9.



1. LEAF EXTRACT 2.WATER EXTRACT OF LATEX
3. ETHYLACETATE EXTRACT OF LATEX
4. ETHYLACETATE EXTRACT OF LATEX PROTECTED BY SODIUM BENZOATE
5. METHANOL EXTRACT OF LATEX
6. HEXANE EXTRACT OF LATEX

Fig 4.9. TLC separation of different extracts.

CHAPTER 5

CONCLUSION

This study shows that all the extracts of *J.curcas L.* is effective against the test organisms like *Streptococcus mutans, E.coli, P. aeruginosa, S. aureas, Proteus and K. pneumonia.* Also the phytochemical analysis revealed the presence of metabolites like tannins, saponins, phenolics, flavonoids, alkaloids and glycosides. Tannins and Saponins are responsible for the antibacterial activity. Also MIC assay revealed the MIC concentrations ranging from 10-640µg/ml for the test organisms. TLC separation revealed the presence of Phenolic acid in the extracts which also possess anti-oxidant activity.

Thus this study shows that *J.curcas L.* is a best source of medicine which can be used in future in all sectors like transportation, agriculture, cosmetics, medicine etc., Also it is distributed worldwide henceforth it can be used effectively and efficiently.

SCOPE FOR FUTURE WORK

Since water extracts of latex found to possess anti-microbial activity against *S.mutans* , the future work is towards formulation of mouthwash liquid with phytochemicals which possess anti-microbial activity.

Also literature says that latex possess a very good anti-coagulation activity henceforth by proper standardization my future work is also towards production of curcain films which can be used for first aid.

APPENDICES

1.APPENDIX I

I. COMPOSITION OF MEDIA

1. NUTRIENT AGAR:

Ingredients	**g/l**
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C) 7.4±0.2	

2. NUTRIENT BROTH:

Ingredients	**g/l**
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Final pH (at 25°C) 7.4±0.2	

3. MUELLER- HINTON AGAR:

Ingredients	**g/l**
Beef, dehydrated infusion of 300 g	2
Casein hydrolysate	17.5
Starch	1.5
Agar	17
Final pH (at 25°C) 7.4±0.2	

2. APPENDIX II

II. COMPOSITION OF SOLUTION

1. MARQUIS REAGENT:

100ml concentrated sulphuric acid is added to 5ml 40% formaldehyde.

2. 80% ETHANOL:

100ml solution containing 80ml absolute ethanol and 20ml distilled water.

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