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Isolation and Quantification of Flavonoid from *Euphorbia antiquorum* Latex and its Antibacterial Studies

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ABSTRACT
Euphorbia antiquorum L. *is conspicuously and regularly represented in the flora of the Rayalaseema region, A.P, India and is used as insecticide, fungicide, anti-hyperglycaemic, cytotoxic agent, for curing skin infections, applied to cosmetic injuries and wounds for speedy healing. E. antiquorum containing latex, distributed in laticiferous tissue. Its extracts containing flavonoids as the principal physiologically active constituents; this class of natural products is becoming the subject of antibacterial activity. The present study is focussed to isolate and quantify latex from phylloclades, roots of E. antiquorum and investigate their antibacterial activities. Antibacterial activity was tested against Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Escherichia coli, Streptococcus anginosus and Bacillus subtilis. Disc method of Bauer-Kirby was found that, flavonoids of E. antiquorum latex were effective against G^{+ve} and G^{-ve} bacterial species. 30 µg/ml concentration of extract is sufficient to constrain bacterial growth. Qualitative analysis of flavonoids and quantitative analysis of total flavonoids has been carried out and the studies revealed that the pure latex of E. antiquorum contained 7.54 mg/g total flavonoid.*

Key words: *E. antiquorum*,Methanol extract, Antibacterial activity, Flavonoids

1. INTRODUCTION

India has a rich source of plants and several plant parts/products are known to exhibit medicinal properties for the treatment of various diseases [1]. Sometimes the presence of secondary metabolites such as flavonoids, antioxidants, tannins etc. in plants strongly supports their use in the treatment of wounds, burns and haemorrhoids in herbal medicine [2-3]. Euphorbiaceae *s.l.*, is one of the largest families of flowering plants, composed of over 300 genera and 8,000 species, grown on wide range of habitats [4]. The implication is that, different Euphorbiaceae species are tend to manufacture a wide range of secondary plant substances to aid response to a disparity of stimuli in their particular habitat [5]. Many cultures around the world have used Euphorbiaceae members for its believed medicinal properties. Secondary metabolites such as saponins, flavonoids and tannins from *E. heterophylla* exhibited good activity against xanthine oxidase enzymes [6]. Species such as *E. polycarpa* and *E. hirta* are used for treatment of different ailments in the ancient Ayurveda system [7]. Even today, many Euphorbiaceae plant concoctions, fresh latex and teas are used in alternative medicine. *E. tirucalli* contains large quantities of terpenes and sterols and known for its curative features against diseases like warts, cancer, gonorrhea, arthritis, asthma, cough, earache, neuralgia, rheumatism, toothache, excrescences, tumours and others [8-9]. *E. thymifolia* is used as an anti-viral against simplex virus-2 [10]. Research has shown that some Euphorbiaceae are actually potent as medicinal plants and their extracts have been isolated and patented as modern drugs.

There has been a growing interest in laticifers and their own metabolism, and although there is still limited information available, the importance of latex has been increasingly recognized and revised [11-12]. Recent studies of lattices have contributed to the understanding of the occurrence and structure of secondary metabolites naturally accumulated in laticifers [13-14]. Latex is exudates which flow throughout the plant in an elongated cell called laticifer. Latex contains a variety of chemicals and proteins, such as various terpenoids, alkaloids, rubber, and cardenolides as well as various proteins and enzymes such as proteases, chitinases, and glucosidases [15]. The latices of *E. antiquorum* L., *E. nerifolia* L and *E. tirucalli* L. are highly valued in Indian medicine, mainly as purgatives, in addition to their other therapeutic

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applications [16]. The latex itself is a secondary metabolite [17] and is mixture of compounds such as phytosterols [18], amine oxidases [19] and as well as many others. The latex has also been widely understood to act as a defense for the plant against generalist herbivores.

Euphorbia antiquorum L. can be found growing up to an altitude of 800 m, wide spread throughout peninsular India. One of the largest armed trees of *Euphorbia* with an average height of 5-7 m has been known to attain gigantic proportions if left undisturbed. The odour of its latex is pungent and lingering. The latex of the plant has been described as having emetic, cleansing, diuretic, as well as toxic properties. From ancient times, many village farmers in India have used the latex of *E. antiquorum* for insect pest control. According to past records the latex contains irritant diterpenes and triterpenes. Flavonoids are ubiquitous in photosynthesising cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases. Increasingly, this class of natural products is becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids possessing antifungal, antiviral and antibacterial activity. The substances (Flavonoids) that can either inhibit the growth of pathogens or kill them or have no or least cytotoxicity to host cells are considered candidates for developing new antibacterial drugs [20]. The present work is mainly focused to isolate and quantify flavonoids from latex of phylloclades and roots of *E. antiquorum* and systematically study their antibacterial properties.

2. EXPERIMENTAL

2. 1. Collection of latex

Latex of *E. antiquorum* was collected from the forest area of Guvalchervu (GPRS: 14017'48.18''N 78034'13.36''Eelev 368m) of the Kadapa District in Andhra Pradesh, India. Quantitative latex yield (4h) among different parts (root and phylloclade) of *E. antiquorum* L., was conducted between 7.30 and 10.00 a.m. in full sun at forest. Cuts were made on the plant bodies with a sharp knife. The cut/slicing immediately oozed the whitish latex but oozing did not persist more than 1h. The collected latex was stored in sample containers and brought to laboratory at room temperature. Because of air interaction, solidified latex was initially weighed (along with moisture content) then extracted with hexane and various solvents for qualitative, quantitative analysis of flavonoids and its antibacterial studies.

2.2. Extraction of flavonoids from latex

The latex of *E. antiquorum* was extracted from the plant and defatted thrice with 5 L n-hexane in 1 L beaker for 15 min. using an Ultra-Turrax at 10000 rpm. The n-hexane extracts were filtered of and the solvent was removed under reduced pressure in a rotavapor (Büchi Labortechnik). 5g of defatted latex was extracted three times with 50 mL 60% methanol in water using an ultra-turrax at 9500 rpm for 10 min. The extracts were filtered, concentrated under reduced pressure and dried in a freeze dryer using a 24 h drying program (30 °C for 8h; 0 °C for 4 h; 10 °C for 6 h and 20 °C for 6 h). Exhaustive extraction of the latex of *E. antiquorum* (10 successive extractions with 80% methanol) has yielded 18% crude extract. The extraction method used in this study has provided an extract containing comparable amount to the exhaustive extraction reported earlier [21]. One gram of concentrated extract was dissolved in 30 ml water and extracted three times with an equal volume of n-butanol. The n-butanol fractions were separated from the aqueous part and the emulsified intermediate layer was concentrated under reduced pressure. The concentrated butanol fraction was suspended in a relatively small volume of methanol (2 ml) and allowed to settle. The un-dissolved part was discarded and the dissolved part was added to a large volume of diethyl ether (40 ml). The precipitate formed was separated by centrifugation at 2000 rpm for 20 min at 5 °C and dried. This extract was used for the qualitative, quantitative analysis of flavonoids and its antibacterial activity.

2.3. Qualitative Analysis of Flavonoids

The flavonoid fraction was analyzed by TLC using Silica Gel G 60 plates developed with benzene: acetic acid: water (125:72:3) for their flavonoids. The spots were visualized by spraying 5% aq.$FeCl_3$ solution followed by heating at 110 ^{0}C. The digital image of TLC plate flavonoid fraction of *E. antiquorum* latex was showed. Quercetin has been used as reference compound in TLC analysis.

2.4. Quantitative Analysis of Flavonoids

Quantitative estimations of flavonoids were determined by using UV-Visible spectrophotometer at the absorption maxima of 420 nm. Particularly, Aluminium chloride colorimetric method was used for flavonoids estimation. The flavonoid fraction of latex (0.5 ml of 1:10 mg^{-1}) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was left at room temperature for 30 min. Then the absorbance of each mixture was measured at 420 nm with a double beam UV/Visible spectrophotometer. The calibration curve was plotted by preparing Quercetin solutions in methanol.

2.5. Reviving Freeze-Dried Bacterial Cultures for Antibacterial Studies

Freeze-dried bacterial cultures namely *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Escherichia coli, Streptococcus anginosus and Bacillus subtilis* (obtained from NCIM Pune) were transferred to 5 ml of nutrient broth and incubated at 35 ^{o}C for 2-8 h. Turbidity of suspension adjusted to yield uniform suspension containing 10^5 to 10^6 Cells/ml.

2.6. Screening for Flavonoid Antibacterial Activity by Filter Paper Method

Flavonoid antimicrobial activity was performed by disc method of Bauer-Kirby [22]. Mueller Hinton Agar (M173) plates were prepared to use in the Bauer-Kirby method for rapidly growing aerobic G^{+ve} and G^{-ve} bacteria. The medium was sterilized at 121 ^{o}C at 15 LBS for 15 min in an autoclave and plates were prepared with a depth of about 4mm. The cultures were transferred to the centre of an agar plate individually and spread evenly over the surface with a sterile bent-glass rod (spreader). 0.2 ml of extract was impregnated onto filter paper discs of 8mm diameter were prepared by using Whatman grade No.1 Filter paper under aseptic conditions with different concentrations of flavonoid extract of latex (i.e. 10, 20, 30 40 and 50 mg/ml), then placed onto a cultured Mueller-Hinton agar plate using a mechanical dispenser or sterile forceps. The plates were incubated for 16 to 18 h, and the diameter of the inhibition zone around the disc was measured. The inhibition zone diameter that is produced will indicate the susceptibility or resistance of a bacterium to the extract. Antibacterial activity can be determined by comparing the zone diameter obtained with the known zone diameter size for susceptibility (penicillin and tetracycline).

3. RESULTS AND DISCUSSION

3.1. Collection of Latex

Several methods have been used for the collection and preservation of latex from *E. antiquorum* (figure1A). Latex could be obtained by making transverse cut in phylloclade or other plant organs or bending or cutting the tops off of the main stem and collecting latex in vials. The amount of latex yield was higher in actively growing herbaceous parts than in mature woody plant parts of *E. antiquorum*. Field observations indicated that root contain less latex than other parts of body of phylloclade. Quantitative comparison of relative latex yield among different parts of *E. antiquorum* L. was shown better yield in phylloclades. The amount of latex was extruded in higher proportions in actively growing phylloclade (3.7 ± 0.6%) parts than in mature woody phylloclades (figure. 1B & 1C). Field observations also indicated that root (figure1D) contain less latex yield (1.1 ± 0.4%), due to less number of laticifers in root system. Also in comparison woody phylloclade portion exuded less latex yield (figure 1C) than younger phylloclade (1.9 ± 0.4%).

3.2. Qualitative Analysis of Flavonoids

From the method of flavonoid isolation (figure 2A), final fractions were found as end product subjected to TLC. After final extraction the stability of the flavonoid in the latex extract preparations was confirmed by TLC comparison. A freshly prepared Quercetin was used as standard and developed a TLC plate (figure 2C). Flavonoid extract has 4.9 ± 0.3% and Quercetin has 5.1 ± 0.1% of RF value (resolution factors). With these results, the end product was considered that the extracted compound is flavonoid.

3.3. Quantitative Analysis of Flavonoids

The concentration of total flavonoids was measured (figure 2D) and found to be 7.542 mg/kg^{-1}. Flavonoids are low molecular weight secondary metabolites i.e., unlike primary metabolites, are not essential for plant survival. Nevertheless, they are bioactive across kingdoms with over 9000 structural variants known. One of their most important roles is to influence the transport of the plant hormone auxin. Quercetin is an important flavonoid and has anticancer, anti-inflammatory, antiviral, fibromyalgia and metabolic syndrome properties. Flavonoid based drugs are frequently used therapeutically in allergic conditions, including asthma hay fever, eczema, and hives. Additional clinical uses include treatment of gout, pancreatitis and prostatitis, which are also in part, inflammatory conditions. The common link is its ability to mediate production and manufacture of pro-inflammatory compounds.

3.4. Antibacterial Activity

The antibacterial activity of flavonoid fraction was studied using filter paper disc method by measuring the diameter of zone of growth inhibition (figure 2B). The results were shown in Table 1. It explores that antibacterial effect of flavonoid fraction of *E. antiquorum* latex had significant differences in their activities depending on the microorganisms tested. Diameters of inhibition zones ranged between 8.6 and 11.3 mm for *Pseudomonas aeruginosa* and 8 to 13 mm for *Klebsiella pneumoniae* were the most susceptible microbes showed with inhibition zones of 16.3 ± 0.5 mm by *Bacillus subtilis*, respectively. *Proteus vulgaris*, *Escherichia coli*, *Streptococcus anginosus* shown moderate inhibition zone of 13 mm from 10µg/ml. The flavonoid antibacterial activity reaches to its maximum at 30µg/ml. Flavonoid fraction of *E. antiquorum* latex has shown high degree of inhibition against most of the selected bacteria. The zone of inhibition increases with increase in

Figure 1. A) Habit of *E. antiquorum* plant. B) Latex collection from younger phylloclades. C) Latex collection from woody phylloclades. D) Latex collection from roots.

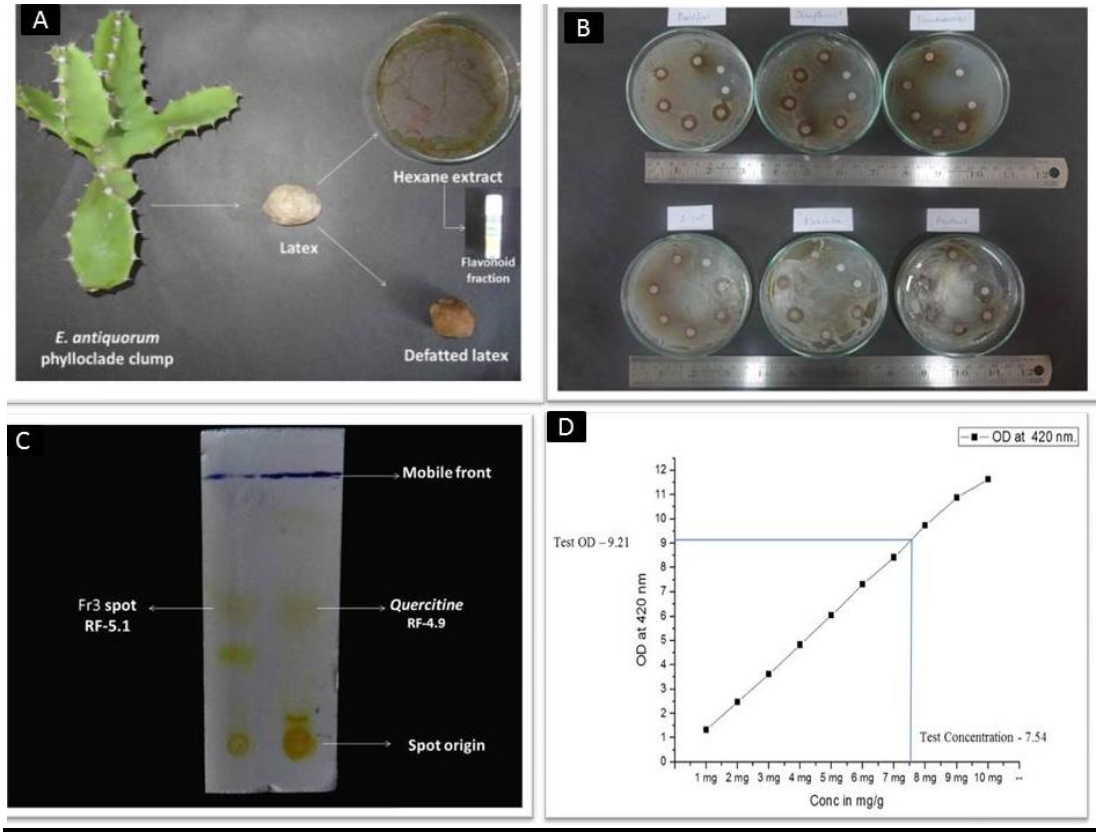

Figure 2. A) Defatted latex extracts and residue of *E. antiquorum*, B) Petri dishes were inoculated with different bacterial strains and inhibition zone of flavonoid fractions were observed. C) TLC of Flavonoid fraction. (Silica gel 60; solvent system: benzene: acetic acid: water (125:72:3); detection: 5% aq.$FeCl_3$). D) Quantitative analysis of flavonoids.

Tables1. Antibacterial activity of *E. antiquorum* latex against different microorganisms

Concentration	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*	*Proteus vulgaris*	*Entamoeba coli*	*Streptococcus aeruginosa*	*Bacillus subtilis*
10µg/ml	8.6±0.5	8±1.0	10±1.0	8±1.0	11±0.0	11.3±0.5
20 µg/ml	9.6±0.5	10±1.0	10.6±0.5	9±0.0	11.6±0.5	10.3±0.5
30 µg/ml	10.6±0.5	13±1.0	12±0.0	10.3±0.5	12±1.0	10.6±0.5
40 µg/ml	12±1.0	13±1.0	12.3±0.5	11.3±0.5	13.6±0.5	8.3±5.5
50 µg/ml	11.3±0.5	13±0.0	13.3±0.5	13±0.0	12.3±0.5	14.3±0.5
Penicillin	0±0.0	7.3±0.5	20±1.0	14.6±0.5	13.6±0.5	16.3±0.5
Streptomycin	19.3±0.5	23±5.1	18±0.0	19.6±0.5	19.6±0.5	20.3±0.5

The data represented above were averages of three replicates with ± standard deviation.

concentration of the extracts up to 30µg/ml indicating that concentration dependent effect. Both gram positive bacteria and Gram negative bacteria are sensitive at all concentrations. However, the antibacterial activity of flavonoid extracts found to exhibit good results in gram negative bacteria viz; *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Escherichia coli*. The gram positive bacteria viz *Streptococcus anginosus* and *Bacillus subtilis* were also susceptible to the flavonoid extracts.The antibacterial activity of flavonoids is being increasingly documented and phytochemical preparations with high flavonoid content have also been reported to exhibit antibacterial activity [23-25]. Many factors are involved in antibacterial disc method and must be carefully controlled. These include size, distribution of the inoculum, incubation period, depth of the agar, diffusion rate of the antibiotic, concentration of antibiotic in the disc and growth rate of the bacterium. If all of these factors are carefully controlled, this type of testing is highly satisfactory for determining the degree of susceptibility of a bacterium to a certain antibiotic. The Bauer-Kirby method is not only restricted to antibiotics, but also used to measure the sensitivity of any microorganism to a variety of antibacterial agents such as sulfonamides and synthetic chemotherapeutics. These observations suggest that bioactive compounds responsible for the antibacterial activity [26].

4. CONCLUSION

The field of antibacterial flavonoid research is widely conflicting. However, several high-quality investigations have examined the relationship between flavonoid and antibacterial activity and these are in close agreement. The results observed were supporting the relation between flavonoids and anti-bacterial activity, and are in line with the therapeutic use of the plant in traditional medicine. In future aspect the defatted hexane extract can be used as renewable energy source. Flavonoid structure elucidation will be studied to support the traditional medicine for developing new drugs.

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****Biographical Sketch***

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