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EXPERIMENTAL INVESTIGATION FOR PEST RESISTANT PROPERTIES OF *Calotropis gigantea*

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ABSTRACT:

Pest resistant properties of *Calotropis gigantea* were tested with the solvent (aqueous, chloroform, ethanol & methanol) extracts of leaves, apical buds and flowers against microbial pest organisms of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas auriginosa*, *Candida albicans* and *Saccharomyces cerevesiae* using agar well diffusion method. The crude solvent extracts exhibited higher pest resistant activities in terms of inhibition zones compared to that of the corresponding soxhlet solvent extracts, which could be due to loss of pest resistant properties of the extracted metabolites by soxhlet extraction procedure involving higher temperatures. The pest resistant properties of methanol extracts, followed by ethanol extracts and aqueous flowers extract were the highest (22-30mm) against *Staphylococcus aureus*, the most purulent bacteria found in the wounds. Similarly methanol flowers extract exhibited better response on *Bacillus subtilis* (20mm) and *Escherichia coli* (21mm), whereas methanol flowers extract exhibited the better response on *Saccharomyces cerevesiae* (24mm). The combined solvent extracts (1:1) of crude solvent extracts and the corresponding soxhelet solvent extracts produced additional pest resistant properties for aqueous leaves extract against *Bacillus subtilis* and *Staphylococcus aureus*. Similarly aqueous flowers extract, chloroform flowers extract and methanol leaves extract have exhibited additional pest resistant activity on some of the bacterial and yeast test microbial organisms, whereas aqueous flowers extract and ethanol flowers extract lost their pest resistant activities against *Staphylococcus aureus* and *Saccharomyces cerevesiae* respectively. The evaluated MIC and MBC values for the *Calotropis gigantea* solvent extracts were better with the values of about 0.2 mg/ml and 0.55 mg/ml respectively on most of the bacterial pests. Hence these experimental investigations produced the evidence and potential of *Calotropis gigantea* as one of the promising alternatives for the extraction and isolation of drugs and pest resistant molecules.

Keywords: *Calotropis gigantea*, solvent extracts, pest resistant properties, flower extracts, microbial pests

[I] INTRODUCTION

Calotropis species, belonging to the family of Asclepiadaceae in plant kingdom, are the well-known plants throughout the tropical world and they are native to the tropical and subtropical parts of Asia and Africa [1]. These plants are

commonly known in English as Giant Milk Weeds or Swallow-worts. This species is one of the special classes of plants that can avoid or repel the grazing animals [2]. The *Calotropis* R. Br genus is known to have two varieties [1, 3], *Calotropis procera* with red or purple flowers and *Calotropis gigantea* with white flowers. Both the plants are reported to possess the similar pharmacological characteristics [1, 4].

Now, in this modern era, pesticides are defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest; whereas the pests are defined as causative living organisms including insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes and microbes, that destroy property, spread disease or are a vector for disease or cause a nuisance (US Environmental Protection Agency). So, it is required to search for the novel and biological sources for the identification, isolation and production of the pest resistant molecules that has the potential to prevent, destroy, repel or mitigate any pest either individually or in combination. In this direction, the systematic screening of antimicrobial plant extracts, especially from medicinal plants, has been carried out, worldwide along with the known phytochemicals to discover novel pest resistance molecules and mechanisms of action for the emerging infectious diseases [5-19].The encouraging efforts across the globe to replace some of the synthetic and polluting antibiotics, pesticides and harmful chemicals existing in the market, by the pest resistant molecules from the secondary metabolites of the many existing medicinal plants, microbial organisms and the animal sources either individually or in combination [20-22] were reported. In this development, *Calotropis* species is also considered to be an ideal plant species as per the recommendations of Singh and Sarathchandra [23] as potential source for the extraction of pest resistant molecules, as this species is perennial, widely distributed, economical & it is adoptable to various adverse ambient conditions. So there had been reports on *Calotropis procera* as sources for the pest resistant molecules and for their extraction with aqueous, polar and non-polar solvents, tested on different pathogenic microbial organisms to start with like, *Escherichiae Coli*, *Bacillus subtilis*, *Candida albians*, *Aspergillus niger*, *Pseudomonas aureginosa*, *Saccharomyces cerevesiae*, *Salmonella typhi*, *Staphylococcus aureus*, *Klebsiella pneumonia* etc. In the similar lines, the present research is carried out to elute the pest resistant properties of *Calotropis gigantea* with the help of solvent extracts of leaves, apical buds and flowers.

[II] MATERIALS AND METHODS

2.1. Preparation of *Calotropis gigantea* solvent extracts

Calotropis gigantea explants of leaves, apical buds and flowers were processed to obtain the dry powder and used them to obtain the respective crude solvent extracts [25] and soxhelet solvent extracts of ethanol, methanol and chloroform.

2.1.1 Preparation of crude solvent extracts

The crude solvent extracts were obtained by dissolving 10% of the respective dry powder of leaves, apical buds and flowers of *Calotropis gigantea* in the selected solvents separately with continuous mixing for 5hrs at ambient conditions and then allowed them to settle for overnight, followed by filteration with 0.45 micron filters to obtain the final filtrates of aqueous leaves crude extract, aqueous apical buds crude extract, aqueous flowers crude extract, ethanol leaves crude extract, ethanol apical buds crude extract, ethanol flowers crude extract, methanol leaves crude extract, methanol apical buds crude extract, methanol flowers crude extract, chloroform leaves crude extract, chloroform apical buds crude extract and chloroform flowers crude extract.

2.1.2 Preparation of soxhelet solvent extracts

The dry powder of leaves (5g), apical buds (2g) and flowers (2g) of *Calotropis gigantea* were taken separately in thimbles and placed them in separate Soxhlet apparatus and extracted the active components with 200ml of ethanol, methanol and chloroform with extraction cycles of 48, 36 & 36 respectively for leaves, apical buds and flowers.

The crude solvent extracts and the corresponding soxhelet solvent extracts were mixed in 1:1 ratio separately and the resultant solvent extracts concentrations were determined by taking a known volume of solvent extract and evaporating the solvents to residues that can be weighed to yield the results shown in Table-1. These solvent extracts were stored in the refrigerator until use.

Table-1. Concentrations of solvent extracts of *Calotropis gigantea*

Name of the solvent extract	Concentration (mg/ml)
Aqueous leaves extract (Aq-L)	22.8
Aqueous apical buds extract (Aq-AB)	20.6
Aqueous flowers extract (Aq-F)	14.6
Chloroform leaves extract (C-L)	19.2
Chloroform apical buds extract (C-AB)	16.4
Chloroform flowers extract (C-F)	20.2
Ethanol leaves extract (E-L)	21.0
Ethanol apical buds extract (E-AB)	23.2
Ethanol flowers extract (E-F)	21.8
Methanol leaves extract (M-L)	29.4
Methanol apical buds extract (M-AB)	26.0
Methanol flowers extract (M-F)	15.2

2.2 Test microbial pest organisms

Pathogenic microbial strains, namely, *Escherichia coli (*MTCC 118), *Staphylococcus aureus* (MTCC 96), *Candida albicans* (MTCC 183) *Saccharomyces cerevesiae* (MTCC 170), *Bacillus subtilis* (MTCC 441) and *Psuedomonas aeruginosa* (MTCC 2295) were used for testing the pest resistant properties of *Calotropis gigantea* solvent extracts. All the microbial strains were obtained from Microbial type culture collection and gene Bank (MTCC), IMTECH, Chandigarh, India; and maintained periodically in the microbiology laboratory, Department of Biotechnology, Sir Padampat Singhania University, Udaipur, Rajasthan, India.

2.3 Assay of the solvent extracts on pathogenic test microorganisms

Assay of both crude solvent extracts and the soxhelet solvent extracts were performed using agar well diffusion method [25-27] where agar plates of Nutrient and YPD media were prepared and autoclaved at 121^{o}C for 15 minutes and these sterile plates were inoculated by spreading 100µl of 0.5 McFarland standard respective overnight grown microbial cultures over the media viz. *Bacillus subtilis* (B.s), *Escherichia coli* (E.c), *Staphylococcus aureus* (S.a) & *Pseudomonas auriginosa* (P.a) on to Nutrient agar plates and *Saccharomyces cerevesiae* (S.c) & *Candida albicans* (C.a) on to YPD agar media surfaces separately by spread plate method. The plates were punched with sterile cork borer to obtain four wells of 4mm diameter at an appropriate equal distance from each other in the center of the plate. For crude solvent extracts and soxhelet solvent extracts, 25µl of solvent extracts of leaves, apical buds & flowers along with respective control solvent were added separately to fill the wells under aseptic conditions for each test organism and then incubated all the plates at their respective optimal culture growth conditions. The best results obtained from both the solvent extracts were listed in the Table-2.

Table-2. Consolidated assay results of *Calotropis gigantea* solvent extracts on pathogenic microorganisms

Solvent Extract	Diameter of Inhibition zone (mm)					
	B.s	E.c	S.a	P.a	C.a	S.c
Aq-L	N	N	N	N	N	N
Aq-AB	8.0	N	N	N	N	7.0
Aq-F	12.0	18.0	24.0	N	N	10.0
C-L	5.0*	7.5	8.0*	10.0*	10.0*	5.0
C-AB	6.5*	15.5	6.0*	6.5*	11.0*	5.0
C-F	N	8.5*	N	6.0*	N	8.5
E-L	10.0	12.0	24.0	7.0	12.0	8.0
E-AB	12.0	14.0	14.0	10.0	14.0	10.0
E-F	8.0	10.0	10.0	6.0	10.0	13.0
M-L	14.0	14.0	30.0	N	N	24.0
M-AB	15.0	16.0	25.0	N	N	10.0
M-F	20.0	21.0	22.0	8.0	6.0	16.0

Note: N - No inhibition zone observed; * the net inhibition zones = (Sample Inhibition zone - Control inhibition zone) + Well diameter (4mm).

2.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Calotropis gigantea* solvent extracts

MIC and MBC of solvent extracts of *Calotropis gigantea* were determined by tube broth dilution assay [28]. A serial 3 fold dilutions of the solvent extracts were prepared and 0.5ml of the solvent extract dilutions were added to sterile MHB broth and YPD broth media of 2.5ml separately. The inoculum of each overnight grown microbial culture was adjusted to 0.5 McFarland standard and added 50µl inoculum of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas auriginosa* to MHB broth with solvent extracts separately, whereas 50µl inoculum of *Candida albicans* and *Saccharomyces cerevesiae* were added to YPD broth media with solvent extracts separately.

Table-3: MIC and MBC results of solvent extracts of *Calotropis gigantea* on the pest microbial organisms

Extract	Values of MIC (MBC) mg/ml on					
	B.s	**E.c**	**S.a**	**P.a**	**C.a**	**S.c**
Aq-L	0.884(2.533)	N	0.281(0.844)	N	N	N
Aq-AB	0.254(0.763)	N	0.254(2.289)	N	0.763 (6.867)	6.870 (20.600)
Aq-F	0.18(0.541)	0.180(0.541)	N	N	N	4.870 (14.600)
C-L	0.237(2.133)	0.237(0.711)	0.237(0.711)	6.400(19.200)	6.400 (19.200)	0.237(0.711)
C-AB	0.202(0.607)	0.202(1.822)	0.202(0.607)	1.822(5.467)	6.400(16.400)	0.202(0.607)
C-F	0.249(0.748)	0.249(0.748)	0.249(0.748)	2.244 (6.733)	0.748 (20.200)	0.249(0.748)
E-L	0.778(2.333)	0.259(2.333)	0.259(0.778)	2.333(7.000)	0.259 (2.333)	0.259(0.778)
E-AB	0.286(0.859)	0.286(2.578)	0.286(0.859)	0.859 (2.578)	0.286 (2.578)	0.286(0.859)
E-F	0.269(2.422)	0.807(2.422)	0.269(0.807)	4.333 (21.800)	0.269 (7.267)	N
M-L	1.089(3.267)	0.363(1.089)	0.363(9.800)	N	0.363 (3.267)	0.363(1.089)
M-AB	0.321(0.963)	0.321(0.963)	0.321(0.963)	N	0.321 (2.889)	0.321(0.963)
M-F	0.188(0.563)	0.188(0.563)	0.188(1.689)	1.689(5.067)	0.188 (1.6689)	0.188(0.563)

These broth samples of *Bacillus subtilis*, *Pseudomonas auriginosa* and *Saccharomyces cerevesiae* were incubated at 30^{o}C and the rest of the culture broth samples at 37^{o}C for 24hrs. *Pseudomonas auriginosa* was incubated for 48hrs. The broth cultures were examined for the macroscopic turbidity and the MIC of solvent extract was determined as the lowest concentration that demonstrated no visible growth. Now the tubes showing no turbidity were diluted 100-fold with drug free MHB and YPD respectively and incubated them at the optimal conditions of the cultures as mentioned above for 48hrs. The lowest concentration of the tube that showed no visible growth in extract free cultivation was considered as the MBC for the respective solvent extract. These MIC and MBC results were shown in Table-3.

[III] RESULTS & DISCUSSION

The crude solvent extracts and soxhelet solvent extracts are of two different ways of preparing solvent extracts, where the crude extraction involved the ambient conditions with mild agitating extraction procedure and on the other hand soxhelet extraction involved the harsher high temperatures for the metabolites extraction. So it may bring out some differences in the quality of metabolites and their efficacy as potential pest resistant molecules against pathogenic organisms. It was also observed [24] that crude extracts of *Calotropis gigantea* had shown better and commendable results on pathogrnic microorganisms compared to soxhelet solvent extracts of the same solvents; which may also indicate the extraction of different components of different concentration in both the solvent extracts. Hence the crude solvent extracts and soxhelet solvent extracts were mixed in equal volumes of the same solvents to investigate their pest resistant properties against the selected pest microbial organisms. Out of the resultant solvent extracts, the concentration of methanol leaves extract had the highest concentration of extract residues followed by methanol apical buds, followed by ethanol apical buds followed by aqueous leaves extract, followed by chloroform flowers extract and so on.

From Table-2, the consolidated results of pest resistant activity of *Calotropis gigantea* in terms of inhibition zones indicate the higher activity on *Staphylococcus aureus* by methanol leaves extract, methanol apical buds extract, ethanol leaves extract and aqueous flowers extract & methanol flowers extract in the decreasing order. Methanol leaves extract had also shown higher pest resistant activity on *Saccharomyces cerevesiae*, and methanol flowers extract had shown higher activity on *Bacillus subtilis* and *Escherichia coli* as well. Similarly, the moderate pest resistant activities were exhibited by aqueous flowers extract on *Bacillus subtilis, Escherichia coli and Saccharomyces cerevesiae;* chloroform leaves extract on *Pseudomonas auriginosa* and *Candida albicans;* chloroform apical buds extract on *Escherichia coli* and *Candida albicans*; ethanol leaves extract on *Bacillus subtilis*, *Escherichia coli* and *Candida albican*; ethanol apical buds extract on all the test organisms; ethanol flowers extract on *Escherichia coli*, *Staphylococcus aureus, Candida albicans* and *Saccharomyces cerevesiae*; methanol leaves extract on *Bacillus subtilis* and *Escherichia coli*; methanol apical buds extract on *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevesiae;* and methanol flowers extracts on *Saccharomyces cerevesiae.* The rest of the pest resistant activities of the solvent extracts on the pest organisms were mild and less than 10mm of inhibition zones. However, the effect of individual aqueous extracts of leaves was not observed on any of the test organisms (Table-2) whereas the combined pest resistant activity of aqueous leaves of crude extract and soxhelet extract on *Bacillus subtilis* and *Staphylococcus aureus* were observed (Table-3). Similarly combined aqueous apical buds extracts had shown the additional pest resistant activity on *Staphylococcus aureus* and *Candida albicans*. Further the combined chloroform flowers had produced an additional pest resistant activity on *Bacillus subtilis, Staphylococcus aureus* and *Candida albicans;* similarly methanol leaves and apical buds extracts had shown an additional pest resistant activity on *Candida albicans*. On the other hand combined aqueous flowers extract had lost its highest pest resistant activity on *Staphylococcus aureus*; whereas the combined ethanol flowers extract had lost its pest resistant activity on *Saccharomyces cerevesiae.*

From Table-3, the minimum inhibitory concentrations (MICs) of active metabolites from different solvent extracts of *Calotropis gigantea* against *Bacillus subtilis, Escherichia coli, Staphylococcus aureu, Psuedomonas aeruginosa, Candida albicans,* and *Saccharomyces cerevesiae* were in the range of (0.180-1.089) mg/ml, (0.180-0.807) mg/ml, (0.188-0.363) mg/ml, (0.859-6.400) mg/ml, (0.188-6.400) mg/ml and (0.188-6.870) mg/ml whereas the minimum bactericidal concentrations (MBCs) were found in the range of (0.541-3.267) mg/ml, (0.541-2.578) mg/ml, (0.607-9.800) mg/ml, (2.578-21.800) mg/ml, (1.669-20.200) mg/ml and (0.563-20.600) mg/ml respectively.

[V] CONCLUSION

The solvent extracts of plant materials may be extracted using different extraction procedures, but then the extracts may dissolve various active ingredients at various concentrations, depending on the extraction conditions. So the extracts may be better extracted with the advanced techniques like freeze drying operating at lower temperatures can preserve the active ingredients in higher concentrations and hence yield the better quality of solvent extracts. However other alternative extraction procedures in terms of economy, availability and novelty may be explored in search of novel extraction procedures.

The combination of extracts from different extraction procedures may also be explored for the better results of plant extracts for pest resistant activities as part of the global search for novel and natural alternatives for controlling pests. The best MIC of solvent extracts was found to be about 0.20mg/ml against most of the pest

microbial organisms, whereas it is about 0.86 mg/ml for *Pseudomonas auriginosa*; which are comparatively better compared to other reported results [29-31] of this *Calotropis* species. However the best minimum bactericidal concentration (MBC) was found to be about 0.55 mg/ml in most of the test organisms where as it was 1.67 mg/ml for *Candida albicans* and 2.58 mg/ml for *Pseudomonas auriginosa*.

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