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Callogenesis and Antibacterial Activity of *Balanites aegyptiaca*

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Abstract

B. aegyptiaca, it is a species of economic and cultural importance in various countries, with diverse uses that include: medicinal, charcoal, pesticides and forage and *in vitro* callus production is important to have many applications in both basic and industrial research on this specie. For the induction of callus, *B. aegyptica* seed cotyledons were surface sterilized with 90% ethanol for 1 minute and cultivated in MS media supplemented with 2,4-D, BAP and NAA. Both the callus and seed were collected and dried in an oven at 40˚C - 45˚C. Cotyledon's seed and callus were grounded into the powdered form using mortar and pestle and stored at room temperature for further use. Five grams (5 g) each of the powder were mixed with 50 ml of the solvents: methanol and n-hexane (1:10) w/v, agitated vigorously and kept on an orbital shaker at 150 rpm for 24 h, then filtered. The extracts of the plant sample were evaluated in agar dilution method which was used to determine the MIC and MBC of the extracts. The auxin NAA in low concentrations (0.5 mg/L) in the presence of a dose of 0.5 mg/L of the cytokinin BAP induced 100% callus formation. The 50 and 100 mg/ml methanolic extracts were more effective than the n-hexane extracts for both the gram-positive and gram-negative bacteria. By callus extracts under 100 and 50 mg/ml reveals that methanolic extracts of callus had the highest zone of inhibition. An effective protocol for callus induction has been developed that can use for germplasm conservation or for genetic engineering. Evidence from the present study revealed both extracts possess strong broad-spectrum antibacterial effect. Therefore, metha-

nolic extract of seed kernel callus of *B. aegyptiaca* can be utilized as a new source of broad spectrum antibacterial drugs for effective control of bacteria related diseases.

Keywords

Antibacterial Activities, Callus Extract, Callus Induction, Minimum Inhibitory Concentration and Seed Cotyledon Extract

1. Introduction

Balanites aegyptiaca L. (Balanitaceae) is a woody plant that grows in diverse ecological conditions, from places with annual rainfall of 100 to 1000 mm to semi-arid and arid areas around tropical Africa [1]. *B. aegyptiaca* is also found in Asia and is grown in the Sahel-Savana regions and regions with less rainfall in the mid-belt areas of Nigeria, Ivory Coast, and Ghana, where it is grown mainly for its fruit [2]. It is a species of economic and cultural importance in various countries, with diverse uses that include: medicinal, charcoal, pesticides and forage [3] [4]. The fruit is 2.5 to 7 cm long, 1.5 to 4 cm in diameter. The immature fruit is green in color and turns yellow when ripe. The seed is a pyrene (stone), 1.5 to 3 cm long, extremely hard, light brown, fibrous, very solid and constitutes up to 60% of the fruit [5].

Plant derivatives are the basis of herbal medicine [6] [7] [8] [9] and many of them have been used as traditional medicines [10] [11] [12], such as *B. aegyptiaca* whose oil contains linear and branched chain alkenes and the seed contains terpenes and sterols (diosgenin), which have the ability to lower liver cholesterol [13] [14]. The aqueous extract of the bark has antifungal activity against *Candida albicans* infections, it is also antiviral and antibacterial [15]. All parts of the plant have some therapeutic use, in Asia and Africa they are used as anthelmintics, antifeedant, molluscicides, antidiabetics and contraceptives [13] [16] [17] [18] [19] [20] that depend on their active phytochemical composition [21] [22]. *B. aegyptiaca* also has a larvicidal effect against mosquito larvae [23]. Among its metabolites, we can mention: flavonol glucoside, balanitisin, diosgenin, deltoin, protodeltoin, alkaloids, balanitoside, steroid saponins, cryptogenin, isorhamnetin-3-O-robinobioside, balanitisin-3 and 6-methyl diosgenin, (25R and S)-spi-rost-5-en-3β-ol, bergapetin, (+) -marmesin and isorhamnetin-3-orutinoside [24] [25] [26] [27].

The massive medicinal use of plant species is limited if there is no culture that supports the demand, against this, tissue culture techniques allow us to produce metabolites of pharmacological interest in the laboratory by culturing callus or cell suspensions, which can lead to industrial-scale production or can be a source of new metabolites that are not present in the wild type [28] [29]. The success of callus culture depends on the secondary metabolite and biomass yield, but can be achieved if the right growth regulators, growing conditions, and nutrients are

available in the right proportion [30] [31]. *In vitro* callus production is an important event in the use of medicinal plants [33] [34] and has many applications in both basic and industrial research [35] [36]. Obtaining secondary metabolites for the manufacture of biocidal, pharmaceutical and food products has given the opportunity for plants to be considered as bioreactors for the production of natural compounds of industrial interest that, under *in vitro* conditions, some researchers have achieved the production of secondary effects in a shorter time through callus induction and the establishment of cell suspensions [28] [29] [36].

Calllogenesis is the obtaining of calluses from their induction by means of growth regulators that allow cell dedifferentiation and obtain totipotent tissue, to achieve this, tests are required regarding the dose of synthetic hormones, for this reason this study aimed to develop a protocol for the induction of *B. aegyptiaca* callus using 6-Benzyl amino purine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D) and *α*-naphthalene acetic acid (NAA) and also to determine the antibacterial activity against three bacteria gram positive bacteria (*Staphylococcus aureus*, *Streptococcus sp.* and *Bacillus subtilis*) and three gram negative bacteria (*Salmonella typhi*, *Escherichia coli* and *Klebsiella pneumoniae*) both from the intact seed cotyledons and from the callus obtained later by the induction protocol that allows the production of antimicrobial metabolites.

2. Materials and Methods

2.1. Samples Collection

B. aegyptica seeds were bought from Yola South market, Adamawa State, Nigeria. The samples were collected in a clean nylon bag and subsequently transported to Plant Tissue Laboratory, Chevron Biotechnology Center, MAUTECH, Yola, Nigeria, where the research was conducted in 2019.

2.2. Media Preparation for Callogenesis

The For the induction of callus, a fixed amount of 1.0 mg/L of 2,4-D [37] was used in all treatments, which resulted from the combination of two doses of the cytokine BAP (0.5 and 1.0 mg/L) and four doses of auxin NAA (0.5, 1, 1.5 and 2 mg/L), additionally a control treatment was added that lacked all the aforementioned regulators. All the treatment regulators were supplemented to the basal medium of Murashige and Skoog. The pH of the media was adjusted to 5.8 and supplemented with 2.5 g of Phytagel was used as solidifying agent. The sterilization of the media was done in an autoclave at 121˚C, 15psi for 15 minutes and media was dispensed into glass bottles to 200 mL.

2.3. Surface's Cotyledons Sterilization

B. aegyptica seed cotyledons were surface sterilize with 90% ethanol for 1 minute, washed 3 times with sterilized distilled water, then with 3.5% aqueous solution of sodium hypochlorite containing few drops of Tween-20 for 10 minutes to break the surface tension of the water and facilitate the cleaning of ex-

ternal contaminants adhering to the surface of the seed, followed by rinsing three times with sterile distilled water.

2.4. Monitoring

The inoculated cotyledons were kept for callus proliferation and their growth was monitored weekly for a period of five weeks. Each treatment was replicated three times. The callus remained proliferating in the medium that achieved their induction and were subcultured every 21 days in fresh medium. They were kept at a temperature of 20˚C with a photoperiod of 12 hours of light and 12 of darkness.

2.5. Preparation of Extract

Both the callus and seed were collected and dried in an oven at 40˚C - 45˚C. They were grounded into the powdered form using mortar and pestle and stored at room temperature for further use. Five grams (5 g) each of the powder were mixed with 50 ml of the solvents: methanol and n-hexane (1:10) w/v, agitated vigorously and kept on an orbital shaker at 150 rpm for 24 h, then filtered. After that, the mixtures were filtered through a double layer of muslin cloth, and then filtered using a Whatman No. 1 filter paper and finally, the filtrate was evaporated until jelly-form using rotary evaporator then kept in a beaker covered with perforated foil paper and placed in an oven at a temperature ranged between 40˚C - 45˚C, to evaporate to powdered form [38] [39].

2.6. Stock Solution

The stock solution was prepared according to Gayathri and Ramesh *et al.* [40] method, 100 mg/ml stock solution of the extracts was made by adding 1 g of the powdered extract to 10 ml of 5% Dimethyl sulfoxide (DMSO), and stored at 4˚C until further use.

2.7. Test Microorganisms

The test organisms were supplied by Bliss Diagnostics Services, Yola. Three gram-positive bacteria: *S. aureus*, *B. subtilis* and *S. sp.*; three gram-negative bacteria: *E. coli*, *K. pneumoniae* and *S. typhi* were used in the study.

2.8. Determination of Antimicrobial Activity

Determination of antimicrobial activity was done according to Reller *et al.* [41] method by using disc diffusion assay. Crude extracts of callus and seed kernel were obtained using methanol and n-hexane as extraction solvents and extracts were prepared into two different concentrations of 100, and a 50 mg/ml. Diffusion disc of approximately 6 mm diameter were prepared from Whatman No. 1 filter paper and was sterilized by autoclaving then by drying in an oven. Thereafter, 10 µl of each concentration of crude extracts was impregnated on separate sterile disc using sterile micropipette tips and stored at 4˚C in separate sterile containers.

2.9. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MBC and MIC of extracts of the plant sample was evaluated in agar dilution method was used to determine the MIC and MBC of the extracts [42]. Equal amount of the stock solution of 100 mg/ml was added into a double strength sterilized molten Mueller Hinton after cooling to 45˚C in water bath followed by serial dilution to obtain 100, 50, 25, 12.50, 6.25, 3.125 and 1.56 mg/ml.

2.10. Statistical Analysis

All the experiments were performed in triplicate, statistically analyzed and expressed as mean ± standard error (SE).

3. Results

3.1. Callus Induction under Different Treatments

Table 1 and Figure 1 show T4 had the least callus formation with 55%, followed by T3 with 66%, then T7 with 89% where T1, T2, T5 and T6 had 100% callus formation but there was no callus formation for the control (T0). No morphogenic responses were observed on cotyledons cultured on MS medium lacking of growth regulators after five weeks of culture. Low concentration auxin for T1, T2, T5 and T6 gave the highest callus induction rate (100%).

3.2. Antibacterial Activities of *B. aegyptiaca* Seed Extract

From Table 2, it was observed that at 50 and 100 mg/ml methanolic extracts were more effective than the n-hexane extracts for both the gram-positive and gram-negative bacteria, (gram-positive bacteria): *S. aureus*, *B. subtilis* and *S. sp*; (gram-negative bacteria): *E. coli*, *K. pneumoniae* and *S. typhi.* The highest zones of inhibition were recorded by methanolic extract of seed kernel at 100 mg/ml were on *E. coli and K. pneumoniae* with 15 ± 0.29 and 15 ± 0.63 mm respectively,

Table 1. Percentage of mean callus induction on the different treatments after 5 weeks.

Treatment (coding)	Concentration of plant growth hormone (mg/L)	Callus induction (%)
T0	0.0 BAP + 0 NAA+ 0 2,4-D	0
T1	0.5 BAP + 0.5 NAA+ 1.0 2,4-D	100
T2	0.5 BAP + 1.0 NAA + 1.0 2,4-D	100
T3	0.5 BAP + 1.5 NAA + 1.0 2,4-D	66
T4	0.5 BAP + 2.0 NAA + 1.0 2,4-D	55
T5	1.0 BAP + 0.5 NAA + 1.0 2,4-D	100
T6	1.0 BAP + 1.0 NAA + 1.0 2,4-D	100
T7	1.0 BAP + 1.5 NAA + 1.0 2,4-D	89
T8	1.0 BAP + 2.0 NAA + 1.0 2,4-D	77

Values are means ± SE from three replicates. BAP = Benzyl aminopurine, 2,4-D = 2,4-Dichlorophenoxyacetic acid, NAA= *α*-naphthaleneacetic acid.

Table 2. Inhibition of different bacteria treated with seed extract of *B. aegyptiaca* after 24 hours of incubation.

Type bacteria	Seed extract	Diameter of zone of inhibition (mm)			MIC and MBC (mg/ml)	
		Solvent	100 mg/ml	50 mg/ml	MIC	MBC
					mg/ml	mg/ml
Gram (+)	*S. aureus*	Methanol	12 ± 0.46	06 ± 0.46	12.5	25
		n-hexane	11 ± 0.87	02 ± 0.61	25	25
	S. sp.	Methanol	12 ± 0.36	05 ± 0.25	12.5	12.5
		n-hexane	11 ± 0.82	02 ± 0.34	12.5	50
	B. subtilis	Methanol	08 ±0.42	03 ± 0.52	25	25
		n-hexane	06 ± 0.27	02 ± 0.32	50	50
Gram (−)	*S. typhi*	Methanol	10 ± 0.42	05 ± 0.55	12.5	25
		n-hexane	08 ± 0.62	01 ± 0.46	25	50
	E. coli	Methanol	15 ± 0.29	08 ± 0.72	12.5	25
		n-hexane	11 ± 0.52	08 ± 0.46	12.5	25
	K. pneumoniae	Methanol	15 ± 0.63	10 ± 0.65	12.5	25
		n-hexane	09 ± 0.75	07 ± 0.58	25	25

Key: Mean of 3 replications ± S.E.M., diameter of zones inhibition excluding diameter of 6 mm disc. Diameter of zone of inhibition of Control (5% DMSO) was 0 mm.

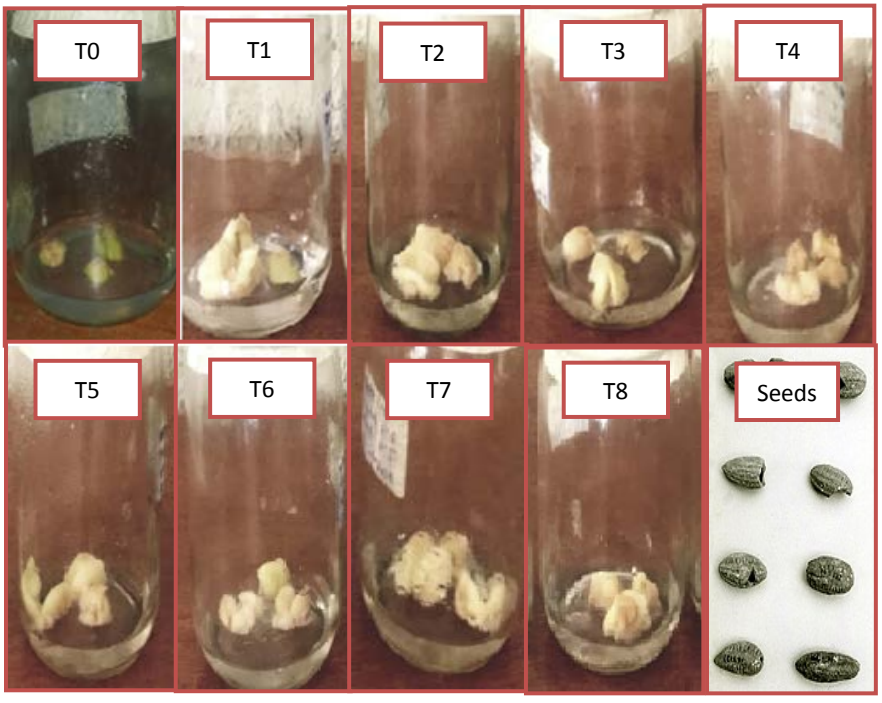

Figure 1. Callus induction on the different treatments after 5 weeks.

while the lowest value (06 ± 0.27 mm) was recorded n-hexane in *B. subtilis*. Generally, the gram-positive bacteria at 100 mg/ml for the callus extracts showed more antibacterial activity for the gram-positive bacteria compared to the seed

cotyledon extracts but *S. spp* showed more susceptibility to the methanolic extracts than *B. aegyptiaca* seed kernel. From the result in Table 2, both the MIC and MBC values ranged from 12.50 to 50.00 mg/ml. Though with lots of fluctuations were observed among the MIC and MBC.

3.3. Antibacterial Activities of *B. aegytiaca* Callus Extract

The diameter of zone of inhibition recorded by callus extracts under 100 and 50 mg/ml reveals methanolic extracts of callus had the highest zone of inhibition (Table 3). From the table, methanolic extract of callus was more effective and higher zone of inhibition in all experiments. The highest zone of inhibition at 100 mg/ml concentration was recorded in E. coli (23 ± 0.23 mm) followed by *S. aureus* with 17 ± 0.25 mm, while lowest zone of inhibition was in *K. pneumonia* (09 ± 0.38 mm) treated with n-hexane extract of *B aegyptiaca* callus. In treatment with 50 mg/ml of callus extract, *E. coli* had the highest zone of inhibition followed by *K. pneumoniae* with 15 ± 0.38 and 13 ± 0.32 mm respectively. The lowest zone of inhibition in treatment with 50 mg/ml of n-hexane extract of callus was in *S. aureus*, *B. subtilis* and *S. typhi* with 02 ± 0.56, 02 ± 0.28 and 02 ± 0.48 mm respectively. Table 3 shows the MIC ranged between 3.125 and 25.00 mg/ml and MBC recorded values between 6.25 to 25.00 mg/ml.

4. Discussion

It was observed that both auxin and cytokinin hormones were necessary to produce

Table 3. Inhibition of different bacteria treated with callus extract of *B. aegyptiaca* after 24 hours of incubation.

Type of bacteria	Callus Extract	Diameter of zone of inhibition (mm)			MIC and MBC (mg/ml)	
		Solvent	100 mg/ml	50 mg/ml	MIC	MBC
					mg/ml	mg/ml
Gram	*S. aureus*	Methanol	17 ± 0.25	05 ± 0.48	6.25	12.5
(+)		n-hexane	15 ± 0.75	02 ± 0.56	12.5	25
	S. spp	Methanol	11 ± 0.34	07 ± 0.37	12.5	12.5
		n-hexane	13 ± 0.76	03 ± 0.28	12.5	25
	B. subtilis	Methanol	10 ± 0.63	04 ± 0.42	25	25
		n-hexane	08 ± 0.23	02 ± 0.28	25	50
Gram	*S. typhi*	Methanol	12 ± 0.56	06 ± 0.68	12.5	25
(−)		n-hexane	09 ± 0.65	02 ± 0.48	25	50
	E. coli	Methanol	23 ± 0.23	15 ± 0.38	3.125	12.5
		n-hexane	13 ± 0.35	06 ± 0.65	12.5	12.5
	K. pneumoniae	Methanol	15 ± 0.12	13 ± 0.32	6.25	6.25
		n-hexane	09 ± 0.38	05 ± 0.78	12.5	12.5

Key: Mean of 3 replications ± S.E.M., diameter of zones inhibition excluding diameter of 6 mm disc. Diameter of zone of inhibition of Control (5% DMSO) was 0 mm.

callus from *Balanites aegyptiaca* seed cotyledon. According to Chapagain *et al.*, frequency of callus induction rate ranges from 55% to 100% on inoculated explant; similar result has also been reported from seed cotyledon of explant which is in agreement with findings of this study [43]. These results were also in line with the report of Sharma *et al.* (2017) and also Sen *et al.* (2014), who stated that 2,4-D in combination with BAP and NAA gave higher formation of callus [44] [45]. According to Phillip *et al.*, (2009) and Abdallah *et al.*, (2012) zone of inhibition equal or above 14 mm is regarded as having high antibacterial property [46] [47]. Interestingly, results from the present study reveals zones of inhibition by callus extract inhibition from 3 trains had values ranging from 15 and 23 mm, while the remaining 3 strains had values between 8 and 13 mm. This suggests that *B. aegyptiaca* seed and callus possess high antibacterial property/potency as antibacterial agent against bacterial pathogens. Similarly, Lystvan *et al.* (2018) and Emmanuel *et al.* (2019) reported that callus can be a source of new metabolites which are not present in the wild type [28] [29]. In this study, MIC and MBC in callus extract were observed to have smaller values than they are in the seed extract. Salvat *et al.* (2004) reported that, low MIC and MBC suggest high antibacterial activity [48], and that high values of MIC and MBC in susceptible bacteria could be as a result of low concentration of active ingredient which may be dependent on extraction method and solvent [22]. The low antibacterial activities observed in n-hexane extracted callus and seed kernel in this study could be attributed to this assertion.

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5. Conclusion

An effective protocol for callus induction has been developed that can use for germplasm conservation or for genetic engineering. Evidence from the present study revealed both extracts possess strong broad-spectrum antibacterial effect. However, the callus extracts generally possessed stronger antibacterial properties/potency compared to the seed cotyledon extracts. Therefore, methanolic ex-

tract of seed kernel callus of *B. aegyptiaca* can be utilized as a new source of broad spectrum antibacterial drugs for effective control of bacteria related diseases. Drugs of this source are generally considered safe, being a product natural/plant base. It can be utilized by pharmaceutical industries for the production of antimicrobials.

Limitations

Some seeds were infected and contaminated during introduction and during cultivation, to avoid this, the explants were stored and constantly checked, checking if they were found free of pathogens before use in subsequent tests.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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