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Research Article
Antimicrobial Activities of *in vitro* and *in vivo* Extract Sources of *Acacia senegal*

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Abstract

Background and Objective: Various research studies reported high contents of bioactive compounds in extracts of *Acacia senegal*. Therefore, this study aims to evaluate the antimicrobial activity of *A. senegal* as affected by the extract source. **Materials and Methods:** Three different type of extract viz. callus, *in vitro* shoot and *in vivo* leaf parts were examined. For callus induction, hypocotyl and cotyledon explants were cultured on Murashige and Skoog (MS) medium fortified with 2, 4-dichlorophenoxyacetic acid (2, 4-D) and naphthalene acetic acid (NAA) (0-3 mg mL^{-1}) alone or in combination with kinetin (Kin) or 6-benzyladenine (BA) (0.5-3 mg mL^{-1}). The three parts of *A. senegal* were macerated in methanol then extracts tested against four pathogenic microbes using agar well diffusion method. **Results:** The best callus induction frequency was attained in hypocotyl explant cultured on MS medium fortified with 3 mg L^{-1} of 2, 4- D and NAA. Callusing was further improved as 2, 4- D and NAA combined with BA and kin lead to high production obtained with 3 mg mL^{-1} 2, 4- D and 1 mg mL^{-1} kin. The results on anti-microbial activity showed substantial growth inhibition by *in vitro* shoot extract followed with *in vivo* leaf extract and then callus extract. The significantly maximum inhibition zones 23 mm were achieved by *in vitro* shoot extract at 50 mg mL^{-1} against *Escherichia coli*. **Conclusion:** Callus induction was accomplished on hypocotyl explant of *A. senegal*. The preliminary antimicrobial screening of *A. senegal* extracts showed variable activities depending on the extract source.

Key words: *Acacia senegal*, antimicrobial activity, callus extract, leaf extract

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Acacia senegal var. *senegal* (L.) Willd. (Leguminosae), is a small (7-15 m height, 1.3 m girth) thorny deciduous multi-functional tree. This legume tree is native to western Sudan, northern Nigeria and the Arabian Peninsula. It is distributed through the dry tropics and subtropics areas of eastern, central and western Africa. The tree, named *Hashab* in Sudan, is well-known as the main source of the world's highest quality exudate i.e., gum Arabic. Although gum Arabic was the famous product from *A. senegal*, different tree parts have been exploited for remedies preparation and other uses. In a survey of traditional uses of *A. senegal* parts in Sudan, root came second after gum and then bark was third in percentage of 58 uses reported[1]. The author also stated that the uses of product of each part depends on the people knowledge and on the way they practice and use. Aerial and root parts has been reportedly used for its astringent, anti-tussive and expect or ant properties. Decoctions, infusion and powder were used to treat bleeding, bronchitis, catarrh, cough, diarrhea, dysentery, gonorrhea, leprosy, typhoid fever, diabetes, upper respiratory tract infections and urinary tract ailments[2,3].

Antibiotic resistance, occurrence of new pathogens and the lack of effective innovative the rapeutics exemplified a serious problem to the medical profession[2]. In addition, the large number of pharmaceuticals synthesized and high quantity consumed symbolized a hazard to human health, e.g., renal failure. Plant extracts enclosed chemically active substances have the potential as efficacy alternative to chemical drugs to treat many human diseases. In the Sudan as in many developing countries, medicinal plants have played an important role in the treatment of diseases especially in rural areas. Nevertheless, the current practices of harvesting, handling, storage and processing of phytopharmaceuticals and using the products for treating ailments is completely based on verbal knowledge. There is no pharmacopoeia or prescribed teaching for the traditional healers and their traditions[3].

Extensive study have been done on secondary metabolites of different parts *A. senegal* by several authors as aerial parts[4], bark[5,6], root[7], leaf[8], stem[9] and seed[10] andpods[11]. These qualitative screenings proved the existence of flavonoids, alkaloids, terpenoids, cardiac glycosides, anthraquinones and volatile oils. In addition to various functions performed by many of these natural products, it was reported to have less side effects on human body or adversarial toxic to environment. Extract of different parts of *A. senegal* were exhibited physiological activity against various human pathogenic micro-organisms as reported by many authors[4-10]. Moreover, bark extract of *A. senegal* was reported to have anti-tuberculosis activity against some Mycobacteria species[12]. According to the best of our knowledge, there is no previous study on anti-microbial activity of extracts of *in vitro* produced parts of *A. senegal*. Moreover, although antimicrobial of *A. senegal* leaves were achieved[8], activity against only one bacterial strain using TLC method was reported and no fungi were studied.

Plant tissue cultures are an attractive alternatives technique to whole plant for the production of high value of active substances. The available literature on culturing explants of *A. senegal* in purpose for callus production, is very limited including cambium[13], immature cotyledon[14] and hypocotyl[15]. Thus, the aim of this research is to establish a callus induction protocol for *A. senegal* and to test the anti-microbial activity of the callus extract in comparison with extracts of plant parts from *in vitro* and *ex vitro* sources.

MATERIALS AND METHODS

***In vivo* plant material:** Fresh bipinnate leaves of *A. senegal* were collected in 2017 at Elobeid area, North Kordofan state, Sudan. Any attached material were detached and the leaf material were washed with distilled water to remove dust.

In vitro plant materials

Establishing culture: Seeds of *A. senegal* were collected in 2005 at El Takamol area, Al Damazin, Blue Nile state, Sudan. The seed were pre-treated with H_2SO_4 (95%) for 10 min before surface sterilization with 20% (v/v) solution of sodium hypochlorite for 10 min then rinsed three times with sterilized distilled water. Disinfected seeds were cultured on Murashige and Skoog (MS) basal medium[16] and incubated under dark at 25±2°C. After 4 days, ≈ 96.9% of cultured seeds were germinated therefore exposed to light under 16 h photoperiod regime for 3 days.

Explants preparation: Three types of explants as hypocotyl (1×1 cm), cotyledons (1-2 cm long) and cotyledonary node (5 cm long) were produced from the 7 days old *in vitro* germinated seedlings. Cotyledons and hypocotyls explants were cultured on MS medium supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) naphthalene acetic acid (NAA) at 1, 2 and 3 mg L^{-1}. To improve callus production, NAA and 2,4-D at 3 mg L^{-1} were combined with kinetin (Kin) or 6-benzyladenine (BA) at 0.5, 1 and 2 mg L^{-1}. Callus index, time required for 100% callusing,

Table 1: Test micro-organisms for *in vitro* antimicrobial screening

Species	Characteristic	Sources
Escherichia coli	Enterobacteriaceae (Gram-negative)	ATCC 25922
Pseudomonas aeruginosa	Non-Enterobacteriaceae (Gram-negative)	ATCC 27853
Staphylococcus aureus	Gram-positive coccus	ATCC 25923
Candida albicans	Fungi	ATCC 7596

callus growth and callus fresh weight were record after 6 weeks of culture. The cotyledonary node explants were cultured in MS medium supplemented with 1 mg L^{-1} BA for 4 weeks as previously reported[17]. Each treatment was repeated 4 times.

Medium and culture conditions: The MS basal medium used in current study was supplemented with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar. The pH of the medium was adjusted to 5.8 before autoclaving at121°C and 15 psi for 15 min. The cultures were incubated at 25±2°C under 16 h photo period provided with cool white fluorescent light (1000 lux).

Extract preparation: Obtained plant material (callus, shoot and leaf) were dried in open air at room temperature (27±2°C) then blended into homogenous powder and keptin air-tight container until use. From each powdered material, a 10 g was soaked in 100 mL of 80% methanol at room temperature for 24 h. The extracts were filtered through What man filter paper No. 1. The filtrates were evaporated to dryness by placing them in a water bath at 40°C overnight. After complete drying of the extracts, weights were attained and stored at 4°C until experiments.

Antimicrobial activity screening: Antimicrobial activity of methanolic extract of the *A. senegal* samples against several organisms (Table 1) were evaluated by the cup-plate agar diffusion method[18]. From the standardized microbial stock suspension 108-109 CFU mL^{-1} (0.5 McFarland scale), 1 mL was taken and mixed thoroughly with 100 mL of molten sterile agar. Then 20 mL of the inoculum were spread on nutrient agar for bacteria and Sabouraud's dextrose agar for fungi. After agar was solidified, 4 cups (each 10 mm in diameter) per plate were cut using a sterile cork borer. The cups were filled with 0.1 mL of *A. senegal* extracts (6.5, 12.5, 25, 50 and 100 mg L^{-1}), ciprofloxacin or itraconazole as positive controls and DMSO as negative control. The plates were kept at room temperature for 2 h to allow extracts to diffuse into the agar. Then all cultures were incubated for 18 h at 37°C and for 24 hat 25°C for the bacteria and fungi, respectively. Three replicates were carried out for each treatment against each of the test organisms. After incubation, the diameters of microbial growth inhibition zones were measured.

Table 2: Effect of different concentrations of auxins on callus induction form hypocotyl explant of *Acacia senegal* after 5 weeks of culture

Auxin (mg L^{-1})	Callus index	Time for 100% callusing (day)
Control	0.00±0.0^{b}	0.0±0.0^{b}
NAA		
1	2.08±0.3^{a}	7.0±0.0^{a}
2	2.42±0.3^{a}	10.1±1.2^{a}
3	3.25±0.4^{a}	10.1±1.2^{a}
2,4-D		
1	2.75±0.3^{a}	8.6±1.0^{a}
2	2.83±0.3^{a}	7.8±0.8^{a}
3	3.33±0.2^{a}	7.0±0.0^{a}

Values are mean±standard errors. Mean values within the column followed by the different letter are significantly different at the 0.05% probability level using Duncan test

Table 3: Effect of combination of NAA and 2, 4-D (3 mg L^{-1}) with kin and BA (0.5, 1 or 2 mg L^{-1}) on callus fresh weight (g) induction form hypocotyl explant of *Acacia senegal* after 5 weeks of culture

		Fresh weight (g)	
Cytokinin	Concentration (mg L^{-1})	NAA	2,4-D
Control	0.0	0.00±0.00^{a}	0.00±0.00^{c}
kin	0.5	0.24±0.04^{a}	0.11±0.02^{bc}
	1.0	0.25±0.15^{a}	0.39±0.01^{a}
	2.0	0.30±0.11^{a}	0.27±0.05^{ab}
BA	0.5	0.20±0.01^{a}	0.35±0.06^{ab}
	1.0	0.16±0.03^{a}	0.20±0.01^{b}
	2.0	0.14±0.02^{a}	0.14±0.02^{bc}

Values are mean±standard errors. Mean values within the column followed by the different letter are significantly different at the 0.05% probability level using Duncan test

RESULTS

Callus induction: No callus response was obtained on cotyledon explants in all auxin treatments. All auxins treatments induced callus on all cultured hypocotyl explants after 15 days of culture. However, 2, 4-D at 3 mg L^{-1} formed the maximum size of callus with index 3.33±0.2 which was not significantly different from 3.25±0.4 obtained with NAA at 3 mg L^{-1} (Table 2). On the other hand, time to 100% callus induction was varies according to auxins concentrations with best time recorded was 7.0±0.0 days both by 1 mg L^{-1} NAA and 3 mg L^{-1} 2, 4-D (Table 2). Growth kinetic of callus showed sigmoid pattern with faster growth by 2, 4-D at 3 mg L^{-1} than NAA at same concentration (Fig. 1). Combination of 2, 4-D at 3 mg L^{-1} with kin at 1 mg L^{-1} improved callus induction significantly with maximum fresh weight obtained was 0.39±0.01 g (Table 3).

Table 4: Antimicrobial activity of methanol extract from *in vitro* and *in vivo* parts of *Acacia senegal*

		Zone of inhibitions (mm)			
Extracts	Concentration (mg L^{-1})	EC	PA	ST	CA
Control	0.00	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}
Ciprofloxacin	0.04 mg mL^{-1}	29.3 ± 0.3^{a}	37.7 ± 0.3^{a}	39.3 ± 0.3^{a}	NT
Itraconazole	0.04 mg mL^{-1}	NT	NT	NT	29.7 ± 0.3^{a}
In vitro shoot	6.25	13.7 ± 0.9^{de}	14.3 ± 0.3^{d}	12.7 ± 0.9^{d}	14.7 ± 0.9^{d}
	12.50	16.7 ± 0.7^{cd}	15.0 ± 1.2^{d}	13.7 ± 0.9^{d}	15.3 ± 0.7^{d}
	25.00	18.7 ± 0.9^{cd}	16.3 ± 0.9^{cd}	17.3 ± 0.9^{c}	17.7 ± 0.9^{cd}
	50.00	23.0 ± 1.0^{b}	18.7 ± 0.9^{c}	19.7 ± 1.2^{b}	21.3 ± 1.2^{bc}
	100.00	23.0 ± 1.5^{b}	19.7 ± 0.9^{bc}	21.3 ± 1.2^{b}	22.7 ± 1.3^{b}
In vivo leaves	6.25	11.7 ± 0.0^{e}	14.3 ± 0.7^{d}	10.7 ± 0.9^{d}	12.3 ± 1.5^{d}
	12.50	12.0 ± 0.9^{e}	15.0 ± 1.2^{d}	11.7 ± 0.7^{d}	14.0 ± 1.0^{d}
	25.00	13.0 ± 1.0^{de}	16.7 ± 0.7^{cd}	13.3 ± 0.9^{d}	15.3 ± 0.9^{d}
	50.00	14.3 ± 1.2^{de}	18.7 ± 0.9^{c}	15.3 ± 0.7^{cd}	16.3 ± 0.9^{cd}
	100.00	19.3 ± 0.7^{c}	22.0 ± 1.5^{b}	17.3 ± 0.7^{c}	18.7 ± 1.3^{c}
Callus	6.25	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}
	12.50	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}
	25.00	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	14.3 ± 0.3^{cd}	0.0 ± 0.0^{e}
	50.00	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	16.7 ± 0.3^{c}	14.7 ± 0.3^{d}
	100.00	16.0 ± 0.6^{d}	14.3 ± 0.7^{d}	19.7 ± 0.3^{b}	17.7 ± 0.3^{cd}

EC: *Escherichia coli*, PA: *Pseudomonas arginosa*, ST: *Staphylococcus aureus*, CA: *Candida albicans*, NT: Not tested, 0: No activity. Values are mean±standard errors

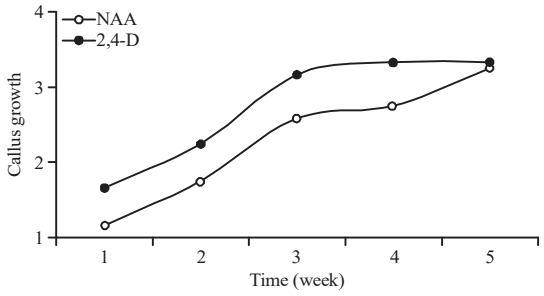

Fig. 1: Growth kinetics of callus induced by 3 mg L^{-1} NAA and 2, 4-D on hypocotyl explant of *Acacia senegal* after 5 weeks of culture

Antimicrobial activity of *A. senegal* extracts: The significantly ($p>0.05$) maximum inhibition zones was recorded by positive controls (Ciprofloxacin and Itraconazole), while negative control (DMSO) showed no activity. The antimicrobial activity of *A. senegal* crude extracts against tested micro-organisms varied considerably between *in vivo* and *in vitro* sources (Table 4). Within each extract type, the inhibition activity was improved with increasing extract concentration, therefore 100 mg L^{-1} have the highest effectiveness. *In vitro* shoot extract showed significantly ($p>0.05$) the highest antimicrobial activity against all evaluated micro-organism except with *P. aeruginosa* where leaf extract appeared more effective. Thus, *in vitro* shoot extract recorded 23.0±1.5, 21.3±1.2 and 22.7±1.3 mm against *E. coli*, *S. aureus* and *C. albicans*, respectively and leaf recorded 22.0±1.5 mm against PA.

Callus extract showed the least inhibition activity as no activity was obtained at the lower concentrations 6.25 and 12.5 mg mL^{-1} against all studied organisms.

Mean values within the column followed by the different letter are significantly different at the 0.05% probability level using Duncan test.

DISCUSSION

Callus induction of *A. senegal* was reported using different types of explants induction including stem cambial zone[13], immature cotyledon[14] and hypocotyl[15]. In the present study, callus induction rate was effected by the type of auxins. Similarly, callus induction percentage was vary according to plant growth regulator type as 100, 58 and 35% were obtained using Zeatin, NAA and 2, 4-D, respectively on immature cotyledon of *A. Senegal*[14]. On the other hand, enhancement of callusing frequency required combining auxin with cytokinin[13], where callus improved on medium fortified with 5 mg L^{-1} 2, 4-D and 2 mg L^{-1} Kin.

The results on anti-microbial activity showed that, the tested micro-organisms revealed variable responses to the different *A. senegal* extracts applied ranged from susceptibility to resistance. Several on antimicrobial studies of *A. senegal* extracts were reported with variations in results on activity due to different plant parts used. Sharma and Kaur[10] reported that *P. aeruginosa* was found resistance to *A. senegal* seed extract while *E. coli* and *S. aureus* were susceptible. Similar to current study, leaf

extract of *A. senegal* was also very effective against *P. aeruginosa* growth[8]. *A. senegal* root extract was found to be very effective against *E. coli*, *S. aureus* and *C. albicans*[7]. In contrary to current study, methanolic extract of *A. nilotica* callus exhibited the highest antimicrobial activity against evaluated micro-organisms compared to bark and leaves extracts[19]. In addition to plant parts effects, the variations on antimicrobial activity of *A. senegal* extracts reported by different studies can also be attributed to the type of solvents used for extraction. For example, Hexane extract of *A. senegal* bark showed substantial activity against *S. aureus* and *C. albicans*. In contrast, the chloroform fraction of the bark was found inactive against *S. aureus*[5]. Moreover, methanolic extract of *A. senegal* bark showed high activity against *E. coli* and *C. albicans* and no activity against *P. aeruginosa*[4].

The antimicrobial activity of *A. senegal* extracts can be accredited to presence of bioactive compounds such as tannins, steroids, cardiac glycosides, flavonoids, saponins, anthraquinone, alkaloids and volatile oil that has been reported by several authors[5-10].

Although *in vitro* shoot extract of *A. senegal* was highly inhabited the growth of most studied microbes, leaves extract also revealed a prominent suppression. Fractionation of methanol crude extract of *A. senegal* should be performed to investigate the antimicrobic effects of each fraction. Thereafter, isolation and identification of bio-active compounds in the active fraction is indispensable.

CONCLUSION

Callus induction was achieved on hypocotyls segments of *Acacia senegal* on MS medium supplemented with 3 mg L^{-1} 2, 4-D combined with 0.5 mg L^{-1} BA. *In vitro* shoot extract displayed remarkable activity against tested micro-organisms compared to *in vivo* leaves. While, callus extract was recorded a very low or no effect on the microbial growth. Antimicrobial activity reported herein indicates presence of chemical constituents in methanol extract, which justify the uses of *A. senegal* in treatment of different pathogenic diseases.

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