Antibacterial and antifungal activity and Bone healing Potency of methanol extract of *Cissus arguta* Hook F

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ABSTRACT

The study was carried out to verify claims for the use of Cissus arguta in the treatment of bone fracture by some bone healing homes in Southern Nigeria. The possible antibacterial, antifungal and bone healing properties by estimation of blood serum calcium levels of Cissus arguta were carried out. The plant parts of C. arguta were extracted using 80% methanol. The extracts were analyzed for phytochemicals, bone healing potency using estimation of blood serum calcium level and also tested for antimicrobial activity using agar well diffusion and serial dilution method respectively.The extract showed the presence of alkaloids, tannins, saponins, flavonoids, terpenoids and eugenols. It also showed positive serum calcium level test which indicates its ability to accelerate bone-fracture healing. The extracts exhibited significant antibacterial and antifungal activity against Staphylococcus aureus, Bacillus subtillis, Enterococcus feacalis, Streptococcus pneumonia, Escherichia coli, Klebsiella pneumonia, Enterobacter species, Proteus mirabilis, Aspergillus flarus, Candida albizans, Microsporuim camb, Tenea pedis and Trichophyton; most of which have been implicated in wound infections. Both the antibacterial and antifungal activity of the extract was significantly higher when compared to standard antimicrobial drugs such as ciprofloxacin-antibacterial and fluconazole -antifungal. This study therefore serves to justify its use for bone healing.

INTRODUCTION

Bone healing or bone fracture healing is a proliferative physiological process in which the body facilitates the repair of bone fracture. The phases of fracture healing include; the reactive phase, reparative and remodeling[1]. *Cissus arguta* a creeping plant found in the rain forest of southern Nigeria, is been extensively utilized locally in the treatment of certain infections such as intense fever, cough, chest pain, inflammation of the lymphatic nodes, blockage of blood vessels, rashes, boils, wounds, skin and sexually transmitted infections, body pains as well as bone related diseases and disorders by herbalist[2]. The plant have been previously indentified in documentaries such Busson[3], Daziels[4] and the Catalogue of life[5] reported its existence in Western Nigeria but not its uses. Commonly reported is the medicinal uses of *Cissus Quadrangularis* which is used in the speed – up of healing of bone fracture, joint and tendon muscle as it is reported to have both analgesic and connective-tissue strengthening processes; it also has high amounts of vitamin C and antioxidants which directly boosts bone reformation and increases bone density[6,7].

One of the greatest challenges in modern times is the alarming rate of mutation especially of pathogenic micro organisms. Most known, once effective and dependable drugs seem to be losing potency with respect to these evolved strains[8]. This puts pressure on existing chemotherapeutic agents (drugs) and calls for increased and concerted research in chemotherapy. Medicinal plants have always been a major source of drugs[9]. Modern research programs to discover and design new drugs are based on plant – derived compounds using medicinal chemistry approach[10].

This study was aimed at determining the phytochemicals, possible antibacterial and

antifungal and bone fracture healing properties of *Cissus arguta* to verify its claim for use in bone fracture healing thus increasing recorded species of plants with medicinal uses..

MATERIALS AND METHODS

Survey Medicinal use in Bone Healing

Visit was paid to bone setting homes in Sapele, Delta State, Nigeria to document the plant materials and possible blending method for preparation of the condiments for bone setting. The procedure was documented.

Plant collection

The whole plant of *Cissus arguta* was harvested between the months of September - October, 2010 from forest reserves at Isihor and Idowinna areas of Benin City, Edo State, Nigeria. The plants were identified at the Department of Plant Biology and Biotechnology, Faculty of Life sciences, University of Benin, Benin City, Nigeria where voucher samples were also deposited. The plant samples collected were separated into the leaves (C1) and stems (C2). These samples were washed under running tap water and air dried in the laboratory for a period of 2 weeks. They were then macerated and further air dried for another period of 8 weeks. The dried samples were pulverized into powder using an electrical shredder and stored in air tight bottles prior to extraction.

Extraction

This was carried out using 80% methanol and Soxhlet apparatus. The solvent was removed using a rotary evaporator fitted with a vacuo pump.

Phytochemical screening

The Plant extracts were screened for the presence of organic compounds using standard procedures by Trease and Evans[11] and Harbone[12].

Experimental animals

A total of twenty one albino rabbits (1.0 - 2.0 kg) of both sexes were obtained from the animal house of the College of Medicine, Ambrose Alli University, Ekpoma, Nigeria. The animals were given two weeks acclimatization in the laboratory before being subjected to experimented protocol. The animals were maintained on standard diet (Ladokun feeds, Ibadan Nigeria) and had access to food and water ad libitum. Animals were housed in a cage with a twelve hour light /dark-circle.

Test for bone healing property of the extract

Popular procedures for assessment of bone healing properties of a substance include radiological and histopathological examinations, estimation of serum calcium levels and formation of calcite crystals. In this study, estimation of serum calcium levels [13] was employed.

Twenty one healthy albino rabbits of both sexes weighing 1 – 2 kg were randomly selected into seven groups, A – G each consisting of three animals. Animals were acclimatized in the laboratory and kept under standard conditions in separate cages. Under anesthesia using intraval sodium 20 mg/kg, i.v), a closed fracture of the right forelimb of each animal was produced by bending either end of the bone in hands. Plaster of Paris bandage was applied over the fractured parts.

The first group (A) received normal saline (10ml/kg to maintain uniformity in volume). Group B to D were given leaf extract of *Cissus arguta* corresponding to 100, 200 and 500 mg/kg respectively every alternate day orally. Groups E to G were given stem extract of the plant corresponding to 100, 200 and 500 mg/kg respectively every alternate day and orally. The duration of this treatment was for a period of 31 days. Health status of the animal was monitored during the treatment period.

On the first day, blood was collected from the Saphenus vein and analysed for estimation of serum calcium level (by O.C.P.C method kit from Linkwell diagnostic laboratories, Benin City, Nigeria). Estimation of serum calcium level was repeated at 10 days interval (i.e at day1, day 11, day 21 and day 31).

Antimicrobial test organisms

A total of 15 microorganisms made up of 5 Gram positive bacteria, 5 Gramm negative bacteria and 5 fungi were used to assess antibacterial and antifungal properties of the crude extracts. They include: *Staphylococcus aureus, Bacillus substilis, Enterococcus feacalis, streptococcus pyogenes and Streptococcus pneumonia* (Gram positive bacteria); *Escherichia coli, Klebsiella Pneumonia, pseudomonas aeuroginosa, Enterobacter species and Proteus mirabilis* (Gramm negative bacteria) and *Aspergillus flarus, Candida albizans, microsporium camb Tenea pedis and Trichophyton* which are fungi. Most of the organisms namely *Streptococcus pyogenes, Enterocossus faecalis, Stappylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pnuemonia, Enterococcus faecalis, Proteus species, Enterobacter species, Candida albizans and Aspergillus flarus* are those implicated in wound infections[14, 15].

All the micro-organisms were obtained from the micro biological cultures of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Nigeria. All cultures were checked for purity and maintained on nutrient or brain heart infusion agar (D_1fco) slabs: slants and stored at 4^oC.

Antibacterial and antifungal test

The agar well diffusion method as well as the serial dilution method as reported by Surgepedia[17](2010) was used to evaluate antibacterial, antifungal and minimum inhibitory concentration of the extracts respectively. The test was carried out alongside using ciproflaxicin for the bacteria and fluconazole for the fungi isolates.

RESULTS AND DISCUSSIONS

Cissus arguta was one of the plants used for treating the bone fracture. The plant stem was pounded with mortar and pestle with the fleshy portion of palm fruits until a good blend was obtained. The blend was then warmed in earthenware pot for about 30mins and allowed to cool. The local healers first arranged the bones as neatly as possible and using lengths of bandage wraped the blend over the area for three or seven days in the first instance depending on the nature of the split (Ebido, 2000).

The results of the phytochemical screening, antibacterial, antifungal of methanol extract of *Cissus arguta* carried out in this study are given in table 1, 2 and 3. The minimum inhibitory concentration

(MIC) and bone healing test are given in figures 1 and 2 respectively.

Table 1 gives the result of the phytochemical analysis carried out on the plant extract. Phytochemical screening of the plant extract revealed the presence of alkaloids, tannins, saponins, flavonoids, glycosides, phenolic compounds, steroids, terpenoids and eugenols in both the leaves and stem extract. These are prerequisite phytochemicals of medicinal plants. Most of these have been associated with anti-microbial, analgesic and bone healing properties.

Both the leaves and the stem extracts exhibited significant antibacterial and antifungal activity against all test organisms as evidenced in their zones of inhibitions (Tables 2 & 3). The highest antimicrobial activity was observed in the stem extract (65.83 $\pm$ 0.14 at 500mg/ml) against *Enterococcus feacalis* while the lowest at that same concentration was also observed for the stem extract against *Candida albizans* (28.5 $\pm$ 0.00). It was also observed that the antimicrobial activity of the extract is dose dependent, increasing with increase dosage and vice versa.

Generally, the stem extract tend to posses more antibacterial activity at all concentrations against Gram positive and Gram negative bacteria when compared to the leave extracts; except for *Bacillus subtilis* and *Pseudomonas aeuroginosa*. On the contrary, the leave extract exhibited higher antifungal properties at all concentrations compared to the stem extract. The disparity in the level of antimicrobial activity of these extract may be due either to different phytochemical composition of the extracts or differences in the concentration of same phytochemicals in the leaves and stem of the plant.

Table 1: Phyto-chemical Screening of Methanol extract of *Cissus arguta*

S/N	Test	C1(Leaves extract)	C2 (Stem extract)
1.	Carbohydrates	+	+
2.	Reducing Sugars	+	+
3.	Carbonyl groups	+	+
4.	Unsaturated compounds	+	+
5.	Alkaloids	+	+
6.	Tannins	+	+
7.	Saponins	+	+
8.	Flavonoids	+	+
9.	Cyanogenetic glycosides	+	+
10	Cardioactive glycosides	+	+
11	Anthraquinones	+	+
12	Anthraquinone glycosides	+	+
13	Phenolic compounds	+	+
14	Eugenol	+	+
15.	Steroids	+	+
16.	Terpenoids	+	+

+ = Presence of phytochemical

Table 2 : Antibacterial activity of *Cissus arguta*

ORGANISMS			**ZONES OF INHIBITION**													
S/N	**NAME**	**Category**	**CONCENTRATIONS C1 (LEAVE EXTRACT) Mg/ml**						**SOLVENT**	**DRUG**	**C2 (STEM EXTRACT) Mg/ml**					
			500	250	100	50	25	7.5		---	500	250	100	50	25	7.5
1	*Staphylococcus aureus*	Gramm +ve bac.	29.70 ± 0.06	20.33 ± 0.92	12.00 ± 0.00	11.17 ± 0.06	-	-	-	28.00 ±0.00	49.50 ± 0.17	25.00 ± 0.00	17.33 ± 0.06	16.00 ± 0.00	14.33 ± 0.22	13.00 ± 0.00
2	*Bacillus subtilis*	Gramm +ve bac.	50.00 ± 0.60	28.00 ± 0.05	18.33 ± 0.06	14.00 ± 0.00	14.00 ± 0.00	14.00 ± 0.00	10.00 ± 0.00	28.00 ± 0.00	49.83 ± 0.39	28.50 ± 0.17	22.00 ± 0.17	19.00 ± 0.17	16.00 ± 0.00	-
3	*Enterococcus feacalis*	Gramm +ve bac.	60.33 ± 0.06	32.00 ± 0.25	23.00 ± 0.06	21.17 ± 0.06	19.00 ± 0.00	15.00 ± 0.00	-	30.00 ± 0.00	65.83 ± 0.14	35.00 ± 0.00	25.50 ± 0.00	18.00 ± 0.17	16.00 ± 0.17	13.00 ± 0.00
4	*Streptococuss pyogenes*	Gramm +ve bac.	28.83 ± 0.06	20.17 ± 0.06	13.33 ± 0.22	12.50 ± 0.00	-	-	-	25.00 ± 0.00	54.17 ± 0.20	26.50 ± 0.17	20.17 ± 0.06	18.33 ± 0.72	15.00 ± 0.00	11.00 ± 0.00
5	*Streptococuss pneumonia*	Gramm +ve bac.	40.00 ± 0.17	28.17 ± 0.06	20.00 ± 0.06	16.17 ± 0.06	14.00 ± 0.00	14.00 ± 0.00	11.00 ± 0.00	25.00 ± 0.06	55.00 ± 0.00	30.17 ± 0.06	25.00 ± 0.17	20.00 ± 0.17	16.33 ± 0.22	14.00 ± 0.00
6	*Escherichia coli*	Gramm -ve bac.	32.33 ± 0.06	18.50 ± 0.00	15.00 ± 0.00	15.17 ± 0.06	12.00 ± 0.00	12.00 ± 0.00	12.00 ± 0.00	19.00 ± 0.00	37.17 ± 0.06	21.33 ± 0.06	17.17 ± 0.06	14.83 ± 0.24	12.00 ± 0.00	12.00 ± 0.00
7	*Klebsiella Pneumonia*	Gramm - ve bac.	32.00 ± 0.17	20.50 ± 0.17	16.00 0+ 0.0	15.00 ± 0.0	15.00 ±0.17	14.00 ± 0.0	12.00 ± 0.0	24.00 ± 0.0	37.00 ± 0.17	21.50 ± 0.17	17.33 ±0.06	16.00 ± 0.00	15.33 ±0.06	12.00 ± 0.00
8	*Pseudomonas aeuroginosa*	Gramm -ve bac.	50.00 ±0.17	31.17 ± 0.06	22.00 ± 0.17	19.50 ± 0.50	17.00 ± 0.00	14.00 ± 0.00	13.00 ±0.00	30.00 ± 0.00	36.83 ± 0.23	21.33 ± 0.06	17.00 ± 0.00	14.50 ± 0.06	14.00 ± 0.00	14.00 ± 0.00
9	*Enterobacter Species*	Gramm -ve bac.	35.33 ± 0.06	22.17 ± 0.06	19.00 ± 0.00	16.50 ± 0.06	13.17 ± 0.06	12.00 ±0.00	11.00 ± 0.00	25.50 ±0.00	41.17 ± 0.06	28.50 ± 0.17	20.50 ± 0.00	17.17 ± 0.06	14.0 ± 0.00	13.33 ± 0.06
10	*Proteus mirabilis*	Gram -ve bac.	33.00 ± 0.00	20.33 ± 0.06	17.33 ± 0.06	15.33 ± 0.06	13.17 ± 0.06	12.00 ± 0.06	12.00 ±0.00	22.5 ± 0.00	41.33 ± 0.06	25.00 ± 0.00	19.33 ± 0.06	16.17 ± 0.06	13.50 ± 0.00	13.17 ± 0.06

Table 3: Antifungal activity of *Cissus arguta*

ORGANISMS			ZONES OF INHIBITION													
S/N	NAME	Category	CONCENTRATIONS C1 (LEAVE EXTARCT) Mg/ml						SOLVENT	DRUG	C2 (STEM EXTRACT) Mg/ml					
			500	250	100	50	25	7.5		--	500	250	100	50	25	7.5
11	*Asperigillus Flarus*	Fungi	52.00 ± 0.17	30.17 ± 0.17	22.00 ± 0.00	19.17 ± 0.06	13.00 ± 0.00	11.00 ± 0.00	-	20.00 ± 0.0	43.00 ± 0.17	25.17 ± 0.06	17.33 ± 0.06	15.33 ± 0.06	11.17 ± 0.06	10.33 ±0.06
12	*Candida albizans*	Fungi	32.17 ± 0.17	18.50 ± 0.17	15.00 ± 0.00	15.00 ± 0.00	14.00 ± 0.00	14.00 ± 0.0	-	19.00 ± 0.0	28.50 ± 0.5	16.50 ± 0.00	13.00 ± 0.00	12.00 ± 0.00	12.00 ± 0.00	-
13	*Microsporium camb*	Fungi	53.33. ± 0.06	33.33 ± 0.06	26.17 ± 0.06	23.00 ± 0.0	20.00 ± 0.00	18.83 ± 0.14	-	25.00 ± 0.0	40.17 ± 0.17	25.17 ± 0.06	20.00 ± 0.00	19.17 ± 0.06	13.00 ± 0.00	-
14	*Tenea pedis*	Fungi	50.00 ± 0.0	31.33 ± 0.06	23.33 ± 0.06	20.50 ± 0.0	17.17 ± 0.06	10.33 ± 0.06	-	25.00 ± 0.00	41.33 ± 0.06	22.50 ± 0.06	20.50 ± 0.00	18.33 ± 0.06	15.50 ± 0.00	11.00 ± 0.00
15	*Trichophyton*	Fungi	49.33 ± 0.06	29.17 ± 0.06	20.33 ± 0.06	18.00 ± 0.00	15.33 ± 0.06	11.33 ± 0.06	-	20.00 ± 0.00	40.17 ± 0.06	22.50 ± 0.00	19.17 ± 0.06	17.00 ± 0.00	14.17 ± 0.06	10.50 ± 0.00

The minimum inhibitory concentration (MIC) of the extract (Fig. 1) also highlights the above mentioned trend. The results revealed variation in the minimum inhibitory concentrations of each extract for given micro-organisms. As already observed, the stem extract exhibit lower inhibitory concentrations for bacteria (except for *Bacillus subtilis*) while the leaves extract showed lower inhibitory values for fungi. The lowest inhibition was 2.5mg/ml. This was observed for stem extract (*E. feacalis, S. pneumonia and K. pneumonia*) as well as leave extract against *A. flarus.* The value of MIC observed for all test organisms was generally low (commonly between 2.5 – 7.5mg/l). Most of these organisms have been implicated in wound and other infections. The activity of the plant extracts even at low concentrations against these organisms justifies its use in treatment of wounds and other infections.

For test organisms, their sensitivity to the leaf extract was in the order *E. feacalis > M. camb > A. flarus > B. subtilis = Tenea = P. aeuroginosa > Trichophyton > S. pneumonia > Enterobacter species > P. mirabilis > E. coli > C. albizains > K. pneumonia > S. aeureus > S. pyogenes.* For the stem extract, microorganism sensitivity was *E. facalis > S. pneumonia > S. pyogenes > B. subtilis. > S. aureus > A. flarus > P. mirabilis > Enterobacter species > M. Camb > E. coli > k. pneumonia > p. aeuroginosa > C. albizans.*

In all, the antimicrobial activity of the plant extract was comparable to standard drugs (fluconazole – antifungal and ciprofloxacin- antibacterial) and was even higher at certain concentrations (500mg and 250 mg/l). The activity of the extract been comparable to standard drugs is significant because, this is a crude extract which is a mixture of many compounds of which the actual concentration of the active ingredient may be very low. Again, some of the components of the crude extract may as well be antagonistic. The ability of the extract even in this crude state to exhibit such level of activity shows that it is of very high potency.

Figure 2 shows the result of serum calcium level test. Serum calcium level can be used as an indicator of a bone healing process (Deka *et al* 1994). Bone healing occurs in three phases: the preparative, reparative and remodeling. The reparative phase involves mineralization in which serum calcium is actively utilized and is accompanied by a subsequent decrease in serum calcium level. This study takes into account, assessment of serum calcium levels at various time intervals (10 days) after fracture. The result of serum calcium levels from the first to thirty-first day after fracture.

On the first day of fracture, the serum calcium level in all the groups was approximately the same. Soliman and Hassan [18] (2004), had previously reported consistency in serum calcium in healthy rabbits. On the 11^{th} day of fracture, all the animal groups revealed a decrease in serum calcium level. However, the decrease was more significant in groups treated with extracts of *Cissus arguta* and this was dose-dependent. Animals treated with stem extracts showed a decrease in serum calcium to a greater extent compared to those treated with leaf extract. On the 21^{st} day of fracture, decrease in the serum calcium level in the control group (28.18%) was still significant; but for groups treated with extracts of *Cissus arguta*, decrease in serum calcium level was relatively very low (0.24% - 4. 26%) and at dosage of 500 mg/ml, serum calcium level remained unchanged. Finally, on the 31^{st} day of fracture, the serum calcium levels in all the experimental animals were again approximately the same.

Fig. 1: Minimum Inhibitory Concentration (MIC) of Methanol extract of *Cissus arguta*

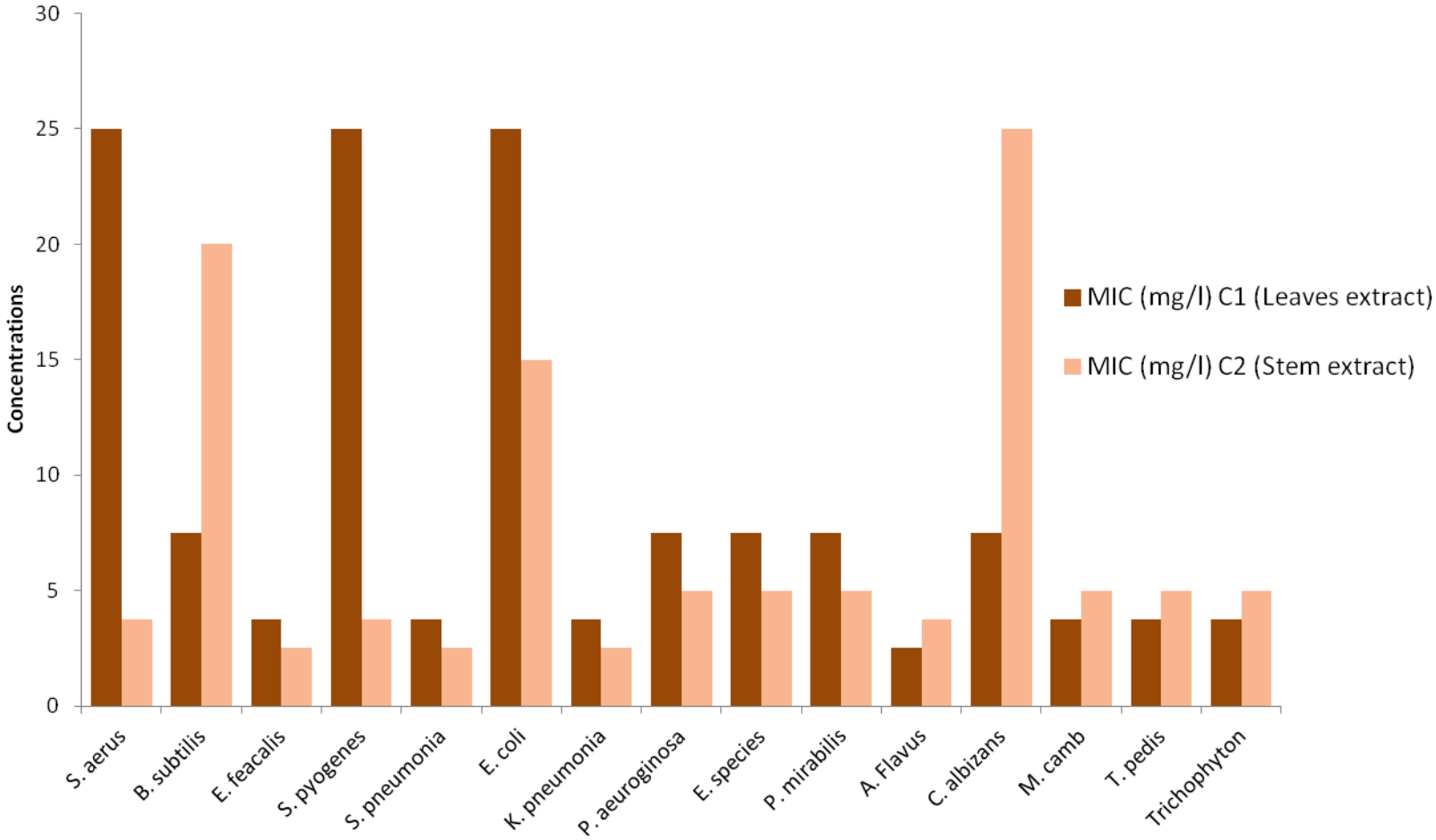
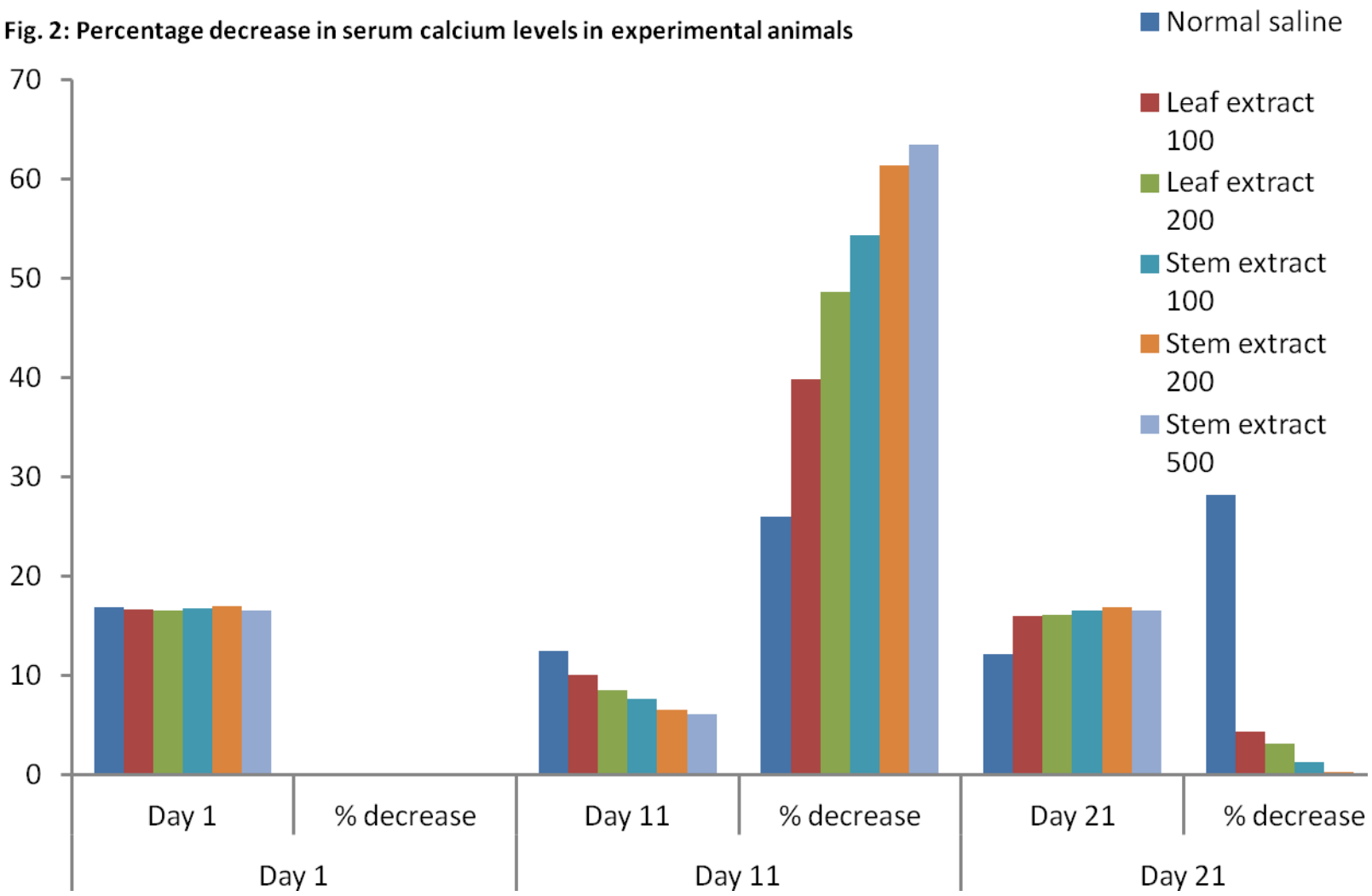


Fig. 2: Percentage decrease in serum calcium levels in experimental animals



A low level of serum calcium in the early stages of fracture healing has already been reported [19](Cohen *et al*, 2007). Furthermore, the calcium of bone callus has been reported to be derived from serum calcium (Deka *et al*, 1994). Thus, serum calcium levels can be used as indicator of bone healing process.
The decrease in the serum calcium to a greater extent in the treated group on the 11^{th} day of fracture may be due to faster healing process with more mobilization of calcium in the formation of bone callus. The significant decrease in serum calcium in the control group still on the 21^{st} day indicates that fracture healing is still going on, in this group. For the treated groups, a relatively very low decrease in serum calcium on the 21^{st} day and unchanged serum calcium level at some instances indicates that fracture healing is nearly complete or complete. On the 31^{st} day, serum calcium levels in all groups had return to normal; showing that, fracture healing had possible been completed in all the groups.

The result of this study, shows that methanol extract of *Cissus arguta* accelerates bone healing process in the experimental animals with fracture healing complete or nearly complete after 21 days.

CONCLUSION

The result of this study shows that, the methanol extract of the plant has both antibacterial and antifungal activity against microorganisms especially those implicated in wound infections. Also, the leaves of the plant possess more of antifungal activity while the stem has more of antibacterial activity. It shows that *Cissus arguta* has the ability to accelerate bone – fracture healing. It also justifies the use of this plant locally in the treatment of certain diseases and wounds.

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