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Evaluation of antibacterial and wound healing activity between field grown and *in vitro* regenerated *Cissus quadrangularis* L.

Hridhya M V, Rinku C P, Aiswariya K S, Vimala Jose*
Research and Post Graduate, Department of Botany, St Thomas' College (Autonomous), University of Calicut, Thrissur, Kerala, India

Abstract
Cissus quadrangularis L. is a perennial shrub with many medicinal properties distributed throughout the tropical region. The primary aim of this work was the *in vitro* regeneration of this plant. The culture was established in MS medium, which showed favourable shoot initiation and multiple shoot formation. The comparative antibacterial activity and wound healing property between field grown and *in vitro* regenerated *Cissus quadrangularis* was attempted. The average diameter of zone of inhibition was greater for field grown plant extract than *in vitro* plant extract. Plant extracts were also used to assess the excision wound healing potential in mice. It can be concluded that field grown plant extracts exhibits comparatively greater antimicrobial and wound healing potential than tissue cultured plant extracts. This provided scientific evidence to the ethnomedicinal uses of *Cissus quadrangularis.*

Keywords: *Cissus quadrangularis*, *in vitro* regeneration, wound healing potential, anti-microbial activity

Introduction

Cissus quadrangularis L. (Vitaceae) is commonly known as 'bone setter' in English and 'Hadjod' in Hindi due to its unique ability to promote the healing of fractured bone. It is a rambling shrub, characterized by a thick quadrangular stem, found throughout the warmer parts of India, Sri Lanka, Malaysia, Thailand and West Africa (Udupa *et al.*, 1970) [28]. The versatile uses and various therapeutic activities have made the plant a valuable medicinal climbing shrub (Kirtikar *et al*., 2000) [13]. The stem is employed for the reatment of eye and ear diseases, irregular menstruation, asthma, piles, tumors, fractures of bones, stomach ulcer, wounds and scurvy. In Cameroon, the whole plant is used in oral re-hydration, while in Africa and Asia the leaf, stem, and root extracts are utilized in the management of various ailments (Chidambara *et al.,* 2003; Das and Sanyal, 1964; Singh *et al*., 1984; Oliver, 1983) [5, 7, 26, 23]. The plant is in great demand for the production of traditional as well as in modern medicines. Its constant use in medicines, scientific studies, food and lack of its conservation strategies has caused its significant depletion from the forests. In *Cissus quarangularis* conventional methods of propagation nvolves seed germination and stem cutting. Both these methods are of limited use when comes to mass propagation of the plant as the seed germination rate was found to be very low and propagation through cuttings limit the number of ropagules. Therefore the application of a reliable, *in vitro* clonal ropagation system would provide an alternate method of propagation to meet the pharmaceutical needs and for ffective conservation of this precious medicinal plant (Malik and Garg, 2012) [16].

Though this plant have been screened cientifically for evaluation of their various medicinal activities in different pharmacological models, the wound healing property of *C. quadrangularis* remains unexplored.

The integrity of the skin is critical for the, maintenance of body temperature, protection, sensation, metabolism and communication (Langoen and Bianhi, 2013). Skin issues, particularly wounds are very common ailments. A wound is a break in the continuity of tissue (Juneja *et al*., 2020) [12]. When there is a restoration of the wounded tissue to normal condition, the wound is considered to be healed (Attama *et al*., 2011) [2]. Therefore present study was conducted to regenerate *Cissus quadrangularis* through tissue culture and to investigate the excision wound healing potential of regenerated plants in comparison with the field grown plants.

Materials and Methods

In vitro plant propagation

Plant material collection

Cissus quadrangularis, used as explant source for *in vitro* culture were maintained in the germplasm conservatory of Kerala Forest Research Institute (KFRI), Thrissur, India, who hold the national mandate for collection and conservation of forest genetic resources.

Inoculation of explants

Nodal segments obtained from field grown plants were washed in tap water and surface sterilized with 0.1% $HgCl_2$ for 3 minutes, washed thrice with sterile distilled water and aseptically transferred to MS medium (Murashige and Skoog, 1962) [20] supplemented with 1mg l^{-1} BA and 2 % sucrose. The pH was adjusted to 5.8 before adding 0.8 % agar. They were maintained in 25 + 2ºC under 12 hrs photoperiod.

Sub culturing and hardening

Plantlets derived from nodal segments were transferred to MS medium supplemented with 0.5 mg l^{-1} BA, 0.5mg l^{-1} IAA and 3% sucrose. The optimum conditions such as 25 ± 2°C temperature, 50% - 60% humidity and a photoperiod of 12 hrs/day were maintained in the culture room. After 15 days, the regenerated shoots were again subcultured on MS media supplemented with different combinations of BA (0.5 mg l^{-1}, 1 mg l^{-1}, 1.5mg l^{-1} and 2 mg l^{-1}), 1mg l^{-1} IAA and 3% sucrose. The rooted plants (10 –15 cm long) were washed under running water to remove traces of agar and transferred to plastic cups containing sterile sand. The cups were kept covered with polythene bags and kept in a shade house. The polythene cover was removed after one month and the plants were transplanted to polythene bags containing sterile potting mixture.

Antibacterial studies

Plant extract preparation

The stem of *Cissus* was cut into pieces and washed in tap water; dried in shade and powdered in mechanical grinder and stored in airtight bottle for future use. The shade dried and finely ground sample (50g), was extracted by different solvents *viz* petroleum ether, chloroform, acetone, methanol, and chloroform in a specific sequence based on increasing polarity. The soxhlet hot extraction procedure for each of the above solvents was run for about 6 hours, until a colourless solvent was seen in the siphon tube, which indicated complete extraction (Luque and Garcia, 1998) [15]. The solvents were removed under reduced pressure and controlled temperature by rotary evaporator. The extracts were dried and stored in a clean glass bottle and kept at 4ºC for further studies on antibacterial screening and wound healing activity. The extracts of the *in vitro* raised plants (50g dried sample) 3 months after transplantation were also obtained in a similar way.

Antibacterial assay

25 ml of nutrient agar media (Hi Media, Mumbai) was poured into sterilized petriplates under sterile conditions and left it to solidify at room temperature. Agar surface of the plates was smeared with a sterile cotton swab of the selected bacterial strain. The culture suspensions from the pure cultures of bacteria *Bacillus subtilis* (MTCC441), *Staphylococcus aureus* (MTCC96), *Escherichia coli* (MTCC443) and *Klebsiella pnuemoniae* (MTCC109) were chosen based on their clinical and pharmacological importance (McCracken and Cowsan, 1983) [19]. The bacterial strains obtained from Institute of Microbial Technology, Chandigarh, India, were used for evaluating antimicrobial activity. Antibacterial activities of plant extracts against four human pathogens were investigated by the agar disc diffusion method (Rios *et al*., 1988; Alzoreky and Nakahara, 2003) [25, 1]. Sterile discs were impregnated with the fractions 20µl of plant extracts (concentration 200mg/ml obtained after soxhlet extraction). *In vitro* and field grown plant extracts were separately used. Later, it was placed equidistantly on the inoculated media and diffusion of solution was allowed to occur for 30 minutes at room temperature. Readymade antibiotic disc (Gentamicin, Hi Media Laboratories, Mumbai) was used as standard and distilled water taken as negative control for sensitivity testing. Plates were inverted and incubated at 37ºC for 24 hours. Three plates were employed per treatment and the average zone of inhibition (ZOI) was measured in mm. Significance levels of standard and treatments were compared with one way ANOVA test using MSTATC software.

Wound healing activity

The male albino Swiss mice were used for the study. They were kept in animal house in standard conditions of temperature (28 ± 2ºC) and relative humidity (46 ± 6%) with 12 hrs light-dark cycle and adequate ventilation. They were provided food (Mice pellets, Excel India Ltd.) and water during the whole period of the experiment. The animals were physically active and were consuming food and water in a regular way. A total of three animals were used for each investigation. The extracts were administered in a single dose by a suitable intubation canula. A period of 24 hrs was allowed between the dosing of each animal. All animals were observed for the body weights, food consumption, etc for fourteen days.

Excision wound model

The mice were anaesthetized prior to creation of the wounds, with 1 ml of intravenous ketamine hydrochloride (10 mg/kg body weight). The dorsal fur of the animal was shaved and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 2.5 cm in width and 0.2 cm depth was created along the markings using toothed forceps, a surgical blade and pointed scissors (Fig.1 A). The entire wound left open (Diwan *et al*., 1982; Patil & Kulkarni, 1984) [9, 24]. All the surgical interventions were carried out under sterile condition. The animals were divided into three groups of three each and the following treatments were given once daily for fourteen days: Group I animals were control group and treated with petroleum ether (5% v/v), Group II animals were treated with petroleum ether extract of field grown *Cissus* plants (100 µl/kg body weight - topically) and Group III animals were treated with petroleum ether extract of *in vitro* raised *Cissus* plants (100 µl/kg body weight - topically). The measurements of the wound areas of the excision wound model were taken on 1st, 5th 10th and 15th day following the initial wound using transparent paper and a permanent marker (Fig.1 B). The recorded wound areas were measured with graph paper. Progressive decrease in the wound size was monitored periodically. Wound closure, epithelialisation time and weight of the tissue were studied. The period of epithelialisation was calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound. The results were expressed as mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA).

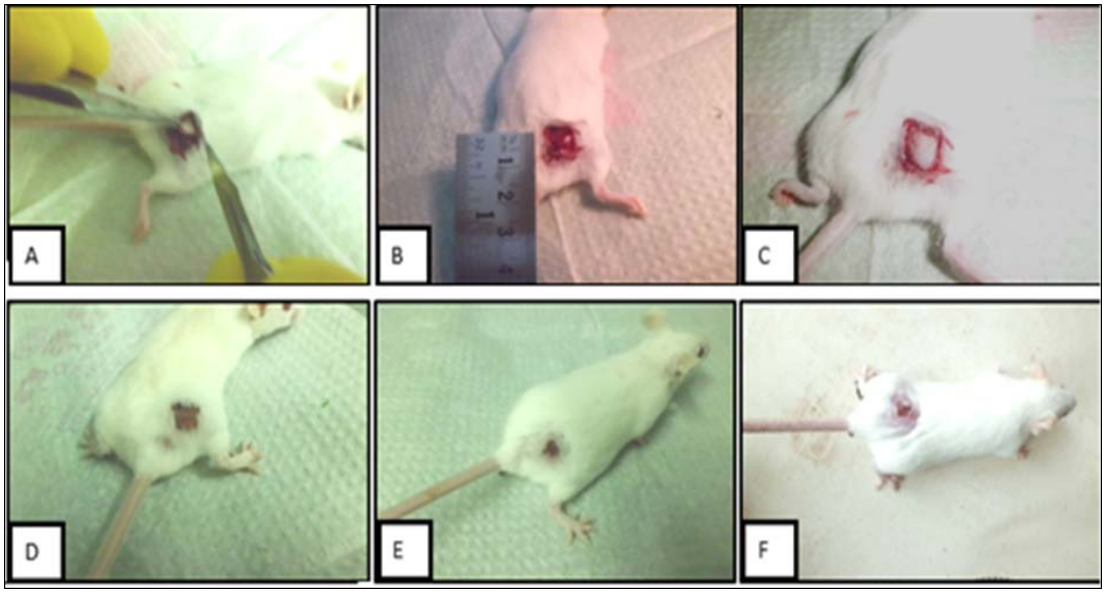

Fig 1: Excision models in mice. A Excision surgery on the dorsal surface. B Measurement of wound area. C Mice used as control in excision model (1st day). D Cootrol mice on 5th day. E Control mice on 10th day. F Control nice on 15th day.

Estimation of Hydroxyproline

In the excision wound model, granulation tissue formed on the wound was excised on the 15th post-operative day and its weight recorded. The tissue was dried in an oven at 60 ºC and the dry weight was again noted and used for the estimation of hydroxyproline. Hydroxyproline present in the neutralized acid hydrolysate was subsequently oxidized by sodium peroxide in presence of copper sulphate followed by complexing with para - dimethyl amino benzaldehyde to develop a pink colour that was measured at 540 nm by spectrophotometer (Neuman and Logan, 1950) [22].

Results and discussion

***In vitro* plant propagation**

The percentage of shoot formation, number of total shoots, and average length of shoot per explant was remarkably influenced by the type and concentration of the growth regulator used (Azad *et al*., 2004) [3]. Shoot initiation was observed within 12 to 16 days of inoculation of explants (Fig. 2 A-F). For multiple shoot induction IAA (1 mg l^{-1}) in combination with different concentrations of BA was tried (Table 1). Among the various BA concentrations tried, the combination of BA (0.5 mg l^{-1}) with IAA could not induce multiple shoot development in nodal explants. Highest percentage of multiple shoot formation was observed in the media supplemented with 1.5 mg l^{-1} BA. The effectiveness of cytokinin especially BA in promoting axillary shoot proliferation in many medicinal plants is well documented e.g. *Stevia rebaudiana* (Debnath, 2008).

Table 1: Multiple shoot regeneration of *Cissus quadrangularis* in different MS media combinations.

Sl. No.	Media	Percentage of multiple shoot formation
1	MS + BA 0.5 mg/l + IAA 1 mg/l	Nil
2	MS + BA 1 mg/l + IAA 1 mg/l	33.3
3	MS + BA 1.5 mg/l + IAA 1 mg/l	66.6
4	MS + BA 2 mg/l + IAA 1 mg/l	44.4

Data scored at the end of 4 weeks in culture. (10 replicates per treatment).

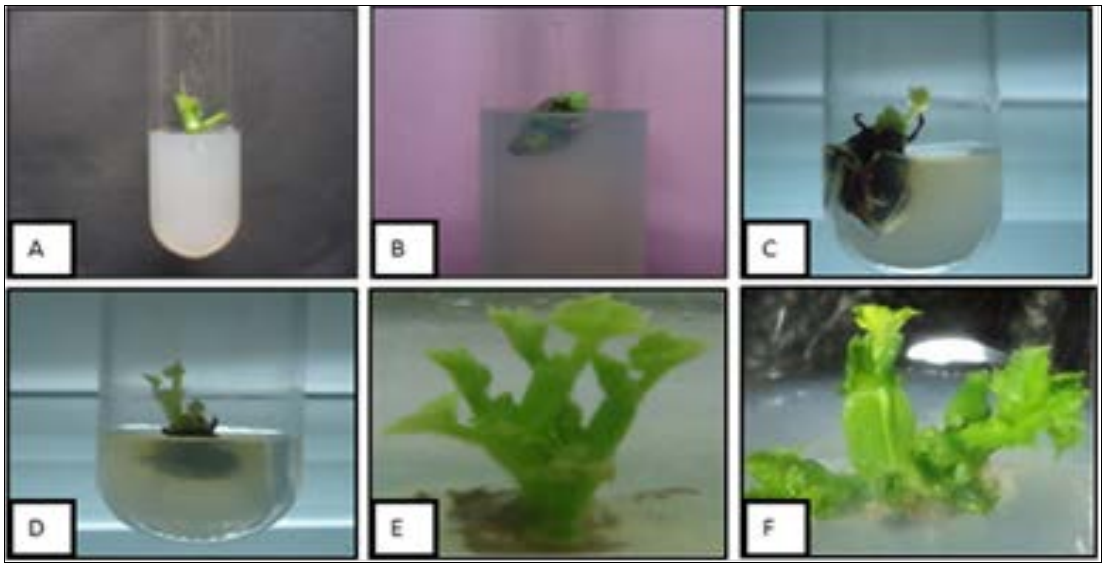

Fig 2: Various stages of shoot initiation in explants. A Explant after inoculation 1.5mg/1 BA+0.5mg/1 IAA. B-C Shoot initiation in the same medium. D-F Various stages of multiple shoot formation in 1.5mg/1 BA medium

Antibacterial studies

The comparative antibacterial screening of the field grown and *in vitro* grown *Cissus* was attempted by using crude plant extracts in four different solvents against four different bacterial strains. And a comparison between field grown and *in vitro* plant extract was done based on the average zone of inhibition. The results of the inhibitory effects of field grown and *in vitro* grown extracts of *Cissus quadrangularis* against *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* indicated that the average zone of inhibition for field grown plant extract was higher than the *in vitro* extract in all the solvents tried (Table 2, Fig. 3). Petroleum ether and methanol field grown extracts showed highest inhibitory effects against *Bacillus, Staphylococcus* and *E.coli*. The results of the inhibitory effects of field grown and *in vitro* grown extracts of *Cissus quadrangularis* against *Klebsiella pneumoniae* indicated no inhibitory activity. Gram negative bacteria *Klebsiella pneumoniae* did not respond to any of the extracts used. Plant extracts were not significant against this bacterium (Fig.2 G & H).

The phytoconstituents alkaloids, glycosides, flavanoids and saponins are antibiotic principles of plants. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens (Maragathavalli *et al*., 2012). Tissue culture often results in somaclonal variations which may negatively influence their inherent medicinal potential (Bairu *et al*., 2011; Currais *et al*., 2013) [4, 6].

Table 2: Inhibitory effects of field grown and *in vitro* grown extracts of *Cissus quadrangularis* against pathogenic microorganisms.

Bacterial isolate		Average zone size (in mm)				
		Petroleum ether	Methanol	Acetone	Chloroform	Gentamicin
E. coli	Field grown	24±1.73*	22.3±3.21*	15±1*	18.33±1.52*	21.33±3.05
	In vitro	16±1.73*	6.3±1.52*	-	-	21.33±1.15
S. aureu	Field grown	26.66±7.63*	28±2*	17.33±1.15*	24±1.73*	23.33±2.08
	In vitro	13.66±3.21*	8.66±1.15*	3.53±2.88*	5±4.35*	24.66±0.577
B.sub tilis	Field grown	30.66±1.15*	28.66±4.61*	21.33±1.15*	16.66±2.88*	24.33±0.577
	In vitro	18.66±2.3*	13±3.4*	6±3.46*	5.33±2.888*	22.66±1.15

Values are expressed as mean ± SD (three replicates per treatment). * indicate the values are significant

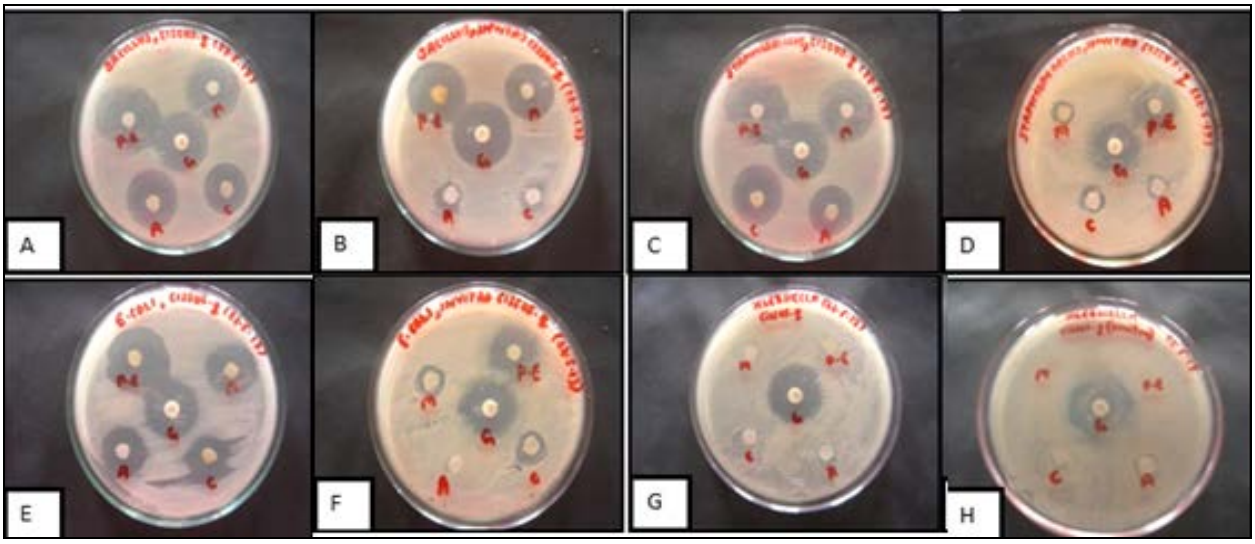

Fig 3: Antibacterial activity of field grown and *In vitro Cissus* plant extract against different bacterial strains. A & B Field grown and *In vitro* plant extract against *Bacillus subtilis*. C & D Field grown & *In vitro* extract against *Staphylococcus aureus*. E & F Field grown & *in vitro* extract against Escherichia coli G & H Field grown & *In vitro extract against Klebsiella* Pneumoniae G-Gentamicin, PE-Petroleum ether, M-Methanol, A-Acetone, C-Chloroform

Wound healing activity

In this investigation field grown and *in vitro* grown *Cissus quadrangularis* extracts were used to assess the wound healing potential in mice when applied topically. There were no significant alterations in water or food consumption, or body weight during the experiment. The body weights were not statistically different from those of the control group.

Wound area

The effect of the field grown and *in vitro* extracts of *Cissus* on excision wound model in mice was tabulated in Table 3. The field grown plant extract (100 µl/kg body weight) treated animals (Fig.4 G-J) showed significant reduction in the wound area ($p < 0.05$), faster rate of epithialization and increased dry weight of the tissue ($p< 0.0\ 5$) when compared with the control group of animals (Fig3.C-F) and *in vitro* regenerated plant extract (Fig.4 K-N).

Table 3: Comparative mean wound area of different animal group recorded on different days after surgery

Animal groups	Mean wound area (in mm)			
	1st day	5th day	10th day	15th day
Control	24.67±0.577	18.0±0	13.0±0	10.0±0
Group 2	24.67±0.577	10.67±0.577	6.16±0.288	2.67±0.577
Group 3	24.67±0.577	17.33±0.288	12.33±0.288	8.16±0.288

Values are expressed as mean ± SD of three rats in each group

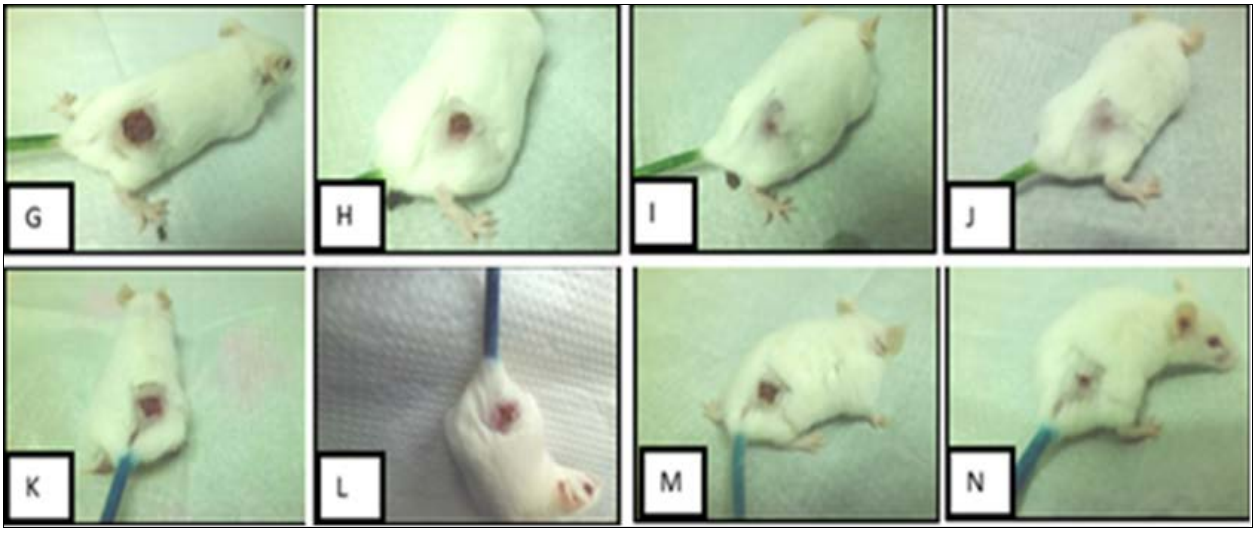

Fig 4: Excision models in mice: G-J Wound treatment with field grown plant extract; G Excision wound on 1st day. H Excision wound on 5th day. I Excision wound on 10th day. J Excision wound on 15th day. K-N Wound treatment with *in vitro* plant extract K Excision wound on 1st day; L Excision wound on 5th day. M Excision wound on 10th day. N Excision wound on 15th day

Estimation of hydroxyproline

There was significant increase in hydroxyproline content in *in vitro* extract treated animals (20 mg/g tissue) as compared to control (18 mg/g tissue). However, more hydroxyproline content was recorded in field grown extract treatment (32 mg/g tissue). The amino acid hydroxyproline is an integral part of the collagen fibre and is used as a biochemical marker for tissue collagen. Hydroxyproline content of tissue was significantly higher in animals treated with field grown extract of *Cissus*. Increased hydroxyproline content indicate increased collagen synthesis. Any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis (Getie *et al.*, 2002) [10]. Antimicrobial agents also provide better and quicker healing by forming a barrier against microbial contamination (Huo *et al*., 2020) [11] Similar types of wound-healing activity were reported on *Vernonia arborea* (Manjunatha *et al.*, 2005) [17]; *Pentas lanceolata* (Nayak *et al.*, 2005) [21] and *Woodfordia fruticosa* (Sonawane *et al*., 2020) [27].

Conclusion

The present study was undertaken to develop an efficient, reliable and reproducible protocol for its clonal propagation of an important medicinal plant *Cissus quadrangulariss* Linn. Under *in vitro* conditions. The data from excision model revealed that *in vitro* grown *Cissus quadrangularis* exhibited no significant wound healing promoting activity. The effect of field grown extracts of *Cissus* (100 µl/kg body weight) resulted with significant wound-healing activity by decreasing period of epithelialization, formation of granulation tissue, synthesis of collagen and by increase in the rate of wound contraction as compared to the control animals. The results of the present investigation showed that C*issus quadrangularis* when grown in natural condition has a definite wound healing action. Although *in vitro* culture techniques offer reliable tools for *ex situ* conservation of the endangered plants, variability may occur as a consequence of tissue culture specific condition. This underlines the fact that plants grown under their natural environmental condition exhibits their full potential of medicinal properties than the plants artificially cultured. Thus the *in situ* conservation of endangered traditional drug plants is of urgent requirement.

Conflict of interest

All authors declare no conflict of interest

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