



## Ayurvedic herbal remedies as bone regenerative materials for surface coating of implants-A preliminary *in-vitro* validation study

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### ABSTRACT

Asthishrinkala and Lakshadi guggulu are traditional ayurvedic herbal constituents widely used in treatment of bone disorders. These herbal constituents improve the bone regenerative capacity by their osteogenic potential. Identify the active constituents and their uses improving the treatment outcome in modern orthopaedic medicine. The aim of the study is to isolate and evaluate the osteogenic activities of aqueous and methanolic extracts of *Cissus Quadrangularis* and Lakshadi guggulu. Various concentrations of aqueous and methanolic extracts of *C. quadrangularis* and Lakshadi Guggulu were subjected to assess the alkaline phosphatase and lactate dehydrogenase activity using spectrophotometric method and their kinetics were determined. The extracts of *C. quadrangularis* and lakshaqi guggulu shows increased alkaline phosphatase and lactate dehydrogenase activity with increasing concentration. Moreover the activity of Lakshadi guggulu methanolic extract was more superior to the *C. Quadrangularis* at respective concentration. Based on the increased alkaline phosphatase and lactate dehydrogenase activity, the *C. Quadrangularis* and Lakshadi guggulu extracts possess significant osteogenic activity which can be used as a competent surface modification material in dental bone healing and orthopaedic titanium implants.

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### INTRODUCTION

Ayurveda a holistic traditional medicinal system followed in Indian subcontinent to treat human disease and increasing health promotions by the help

of Indian herbs. These herbal formulations contain variety of medicinal compounds which improve the health status and alleviating human disorders. Osteoporosis is a phenomenon where weaken and porous bones are formed by decreasing the bone density. Decreased bone mass increases the risk of bone and joint related disorder which is most frequent health illness among the women and old aged peoples and improving bone intensity will reduce these bone related disorders (Putnam *et al.*, 2007). Asthishrinkala' or 'hadjod' a Indian subcontinent vine family herb scientifically known as *Cissus quadrangularis*, widely present in Africa and South Asia which are used as a one of the remedy for bone and joint related disorders (Pansare and Chandil, 2019; Shah, 2011). Lakshadi guggulu a polyherbal mixture with active herbal constituents like Ashwagandha, Asthishrinkala, Nagabala, Arjuna, Laksha

in a specific concentration where listed in Table 1. Asthishrinkala and Lakshadi Guggulu are the two well-known ayurvedic formulation widely used for hastening bone fractures. Moreover, these formulations possess anti-inflammatory and analgesic properties which reduce the burden of bone and joint related disorder (Banu *et al.*, 2012). In addition, modern research findings also demonstrate methanolic extract of *Cissus quadrangularis* prevent osteoporosis which owing to the osteogenic potential of the herbs (Potu *et al.*, 2010, 2009).

Endosseous titanium implants are one of the most accepted dental prosthesis where the damaged teeth are replacement by artificial one where the titanium implants are integrated into the jawbone with the integration time of three to six months. However, titanium implants are successful treatment option for dental and orthopaedic implants, it is important to reduce the implant period with increased osseointegration success ratio (Annunziata and Guida, 2015). Recently, surface modification of titanium implants with osteogenic materials like bone morphogenic protein and tricalcium phosphate reduce the healing time with increased osseointegration. In addition, hydrophilicity of titanium surfaces enhances better osseointegration with reduced implant period (Rupp *et al.*, 2014). However, the major drawback of these surfaces modified implants is expensive and it is not affordable to common people and it is necessary to identify cost effective surface modification materials which increase the osseointegration. Hence, the authors tried to check the feasibility of *C. quadrangularis* and Lakshadi Guggulu as an alternate surface modification material which increase titanium osseointegration. Substance with positive alkaline phosphatase and lactate dehydrogenase activity has a potential to bone reproductive maturation. In the present study, the authors evaluate and compare the *in-vitro* osteogenic potential of *Cissus quadrangularis* and Lakshadi guggulu by assessing the alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activity of aqueous and methanolic extract.

## MATERIALS AND METHODS

### Aqueous and Methanolic Extract Preparation

Freshly cut stem specimen of *Cissus quarangularis* were collected and authenticated from botanical experts. Collected stem specimens were cleaned and air dried at 40°C. Grinded the air dried stem specimen to make a fine powder and it was subjected by using water and methanol as a extraction solvent under Soxhlet extractor. Further the prepared extracts were concentrated at 40°C at rotary

evaporator to get the dry mass of extract. Similarly, the aqueous and methanolic extract was also prepared from freshly prepared Lakshadi Guggulu. The prepared extract was named according to the type of solvent used for extract preparation. Aqueous extract of *Cissus quadrangularis* (P1AE); Methanolic extract of *Cissus quadrangularis* (P2ME); Aqueous extract of *Lakshadi guggulu* (GAE); Methanolic extract of *Lakshadi guggulu* (GME).

### Alkaline Phosphatase and Lactate Dehydrogenase Assay

Prepared extracts were diluted into different concentration ranging from 10-320 mg/L. The diluted extracts were tested with commercially available ALP and LDH testing kits procured from Agappe diagnostics and the absorbance were recorded to determine the enzyme activity and their kinetics were determined as per the protocol mentioned in the assay kit. On days 7 and 14, cellular alkaline phosphatase (ALP) activity was measured. Cell medium was removed, cells were washed with alkaline buffer solution (5 M NaCl, 1 M Tris-Cl pH 9.5, 1 M MgCl<sub>2</sub>), then 250  $\mu$ L of 0.1% Triton X-100 in alkaline buffer was added. Plates were stored at -80 °C and subjected to three freeze-thaw cycles to fully lyse all cells. Once defrosted, 50  $\mu$ L of each lysate was transferred, in duplicate, into a 96-well assay plate and supplemented with 200  $\mu$ L of test solution made of alkaline buffer solution and p-nitrophenyl phosphate substrate (both from Sigma-Aldrich, U.K.). Standards were prepared from p-nitrophenol diluted with alkaline buffer solution. Each plate was then covered in foil (to protect from light) and incubated for 30 min at 37°C. A total of 50  $\mu$ L of stop solution (3 M NaOH) was added to the wells to terminate the reaction and absorbance was then read at 405 nm using a Tecan GENios microplate reader A-5082, (Tecan, Grodig, Austria). Finally, a PicoGreen assay (Thermo Fisher Scientific, UK) was conducted on the same cell lysates according to manufacturer's protocol to measure the amount of dsDNA. This allowed ALP activity to be normalised to cell number.

### Statistical Analysis

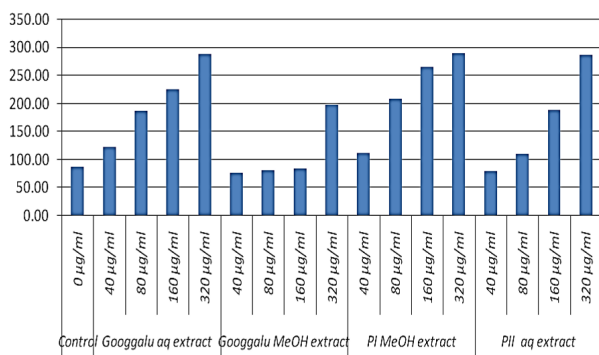
All the obtained results were processed in SPSS/16 statistical analysis and presented as Mean  $\pm$  SD and statistically significant was considered at P value is less than 0.05 by hypothesis testing method which includes ANOVA followed by post hoc analysis.

## RESULTS

### Effect of Extracts on ALP Activity

Figure 1 shows the ALP activity of the isolated aque-

ous and methanolic extract of *Cissus quadrangularis* and *Lakshadi guggulu*. The activity of ALP at concentration below 40  $\mu\text{g/ml}$  was not discernable. However, the activities of higher concentrations were increased in response to their extract concentration. Moreover, the aqueous extract of *Lakshadi Guggulu* showed maximum activity at 320  $\mu\text{g/ml}$  when compared to methanolic extract of *C. Quadrangularis*.



**Figure 1: ALP activity of different concentration of aqueous and methanolic of *Cissus quadrangularis* and Lakshadi guggulu extract. Represented values are mean  $\pm$  SD for 6 independent experiments**

#### Effect of Extracts on LDH Activity

Tables 2 and 3 show the LDH activity of *Cissus quadrangularis* and Lakshadi guggulu extract respectively. Where both isolated aqueous and methanolic extract of *Cissus quadrangularis* and Lakshadi guggulu shows an increased LDH activity in respective of their concentration. Moreover, methanolic extract of Lakshadi Guggulu has increased LDH activity when compared to other extracts.

Based on these observation and statistical analysis, there is no any significant difference between all the four isolated extract. Further the study describes both isolated extracts of *Cissus quadrangularis* and lakshadi guggulu have similar osteogenic potential.

#### DISCUSSION

Ayurvedic medicinal system is one of the traditional health care system in India by using various plants parts such as leaves, barks in the form of crude extract such as tinctures and powders. These plant derived natural products possess variety of pharmacological activities in human pathophysiological conditions (Ramana et al., 2018). Recently, researchers identified constituents of modern medicine which are similar to the active constituents present in the traditional medicine which increasing interest over today's modern medicine. These investigations evoke the knowl-

edge of traditional medicine which encourages developing drugs from the ayurveda herbal plant. The research works focus on these active plant ingredients as a therapeutic agent in numerous life threatening diseases such as cancer etc.,

Recently the herb of *Cissus quadrangularis* has increasing importance in drug discovery due to its variety of biological actions such as anti-inflammatory, anti-osteoporotic properties. Moreover, the compositions of the *Cissus quadrangularis* extract contain hydroxyapatite which is present in mineralized body tissues like bone and teeth. This hydroxyapatite is important for bone regeneration. Calcium and vitamin D are the other important constituents present in *Cissus quadrangularis*. In the present study, the aqueous and methanolic extract of *Cissus quadrangularis* and lakshadi guggulu shows its potential for bone regeneration by increasing alkaline phosphatase and lactate dehydrogenase activity in a concentration dependent manner with an optimal concentration at 120mg/dl. Recently, *Cissus quadrangularis* has been widely used in mandibular fractures repair which reduces fracture healing time through down regulate the inflammation and its associated pain. Moreover it increases the strength of callous which supports that *Cissus quadrangularis* as a healing assistance for fracture (Shah et al., 2015; Singh et al., 2011). Moreover, lakshadi guggulu a polyherbal mixture widely used traditionally as a bone setter which cures fracture by reduced swelling and pain (Mohammad et al., 2014). This beneficial effect of this lakshadi guggulu was mainly due to *Cissus quadrangularis* (Sharma et al., 2007). Moreover, the remaining ingredients assist fracture healing by reducing pain and inflammation (Samarasinghe et al., 2014; Rajoria et al., 2010).

Dental implant is a treatment method to replace the missing teeth through a titanium implant. The success and the duration of a treatment lie on the osseointegration of the implant on the bone surface which is popularly known as bone to implant contact (BIC). Depending upon the bone to implant contact the duration of the healing time and the osseointegration rate can varies from three to six month. Researcher identified that, surface wettable or hydrophilic through surface modified implants can increase the BIC and osseointegration which reduce the normal implant period. *Cissus quadrangularis* have been widely used in implant dentistry as a bone regenerative and periodontal surgery. Validating the comparative products with *Cissus quadrangularis* should improve the therapeutic knowledge of these indigenous herbal products (Lal and Dixit, 2012; Jain et al., 2008).

**Table 1: Constituents and their compositions of Lakshadi Guggulu**

Common name	Scientific name	Parts in mixture
Laksha	<i>Laccifer lacca</i>	1
Ashwagandha	<i>Withania somnifera</i>	1
Hadjod	<i>Cissus quadrangularis</i>	1
Arjuna	<i>Terminalia arjuna</i>	1
Nagabala	<i>Grewia populifolia</i>	1
Shudh guggulu	<i>Commiphora mukul</i>	5

**Table 2: LDH activity of different concentration of *Cissus quadrangularis* extracts**

Test sample	Conc. ( $\mu\text{g/ml}$ )	Absorbance	LDH activity (U/L)
CQ methanolic extract (P1ME)	0	0.1092	1750.48
	10	0.1143	1832.23*
	20	0.1252	2006.96**
	40	0.1314	2106.34**
	80	0.1384	2218.55***
	160	0.1407	2255.42***
	320	0.1426	2285.88***
CQ aqueous extract (P2AE)	0	0.1083	1736.05
	10	0.1127	1806.58*
	20	0.1159	1857.88*
	40	0.1292	2071.08**
	80	0.1353	2168.86***
	160	0.1396	2237.79***
	320	0.1412	2263.44***

Represented values are Mean  $\pm$  SD for 6 independent experiments. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001 statistically significant as compare with negative control

**Table 3: LDH activity of different concentration of Lakshadi guggulu extract**

Test sample	Conc. ( $\mu\text{g/ml}$ )	Absorbance	LDH activity (U/L)
LG aqueous extract(GAE)	0	0.1107	1774.52
	10	0.1156	1853.07*
	20	0.1175	1883.53*
	40	0.1297	2079.09**
	80	0.1322	2119.17*v
	160	0.1368	2192.90**
	320	0.1413	2265.04***
LG Methanolic extract(GME)	0	0.1152	1846.66
	10	0.1187	1902.76*
	20	0.1264	2026.19**
	40	0.1316	2109.55**
	80	0.1395	2236.19***
	160	0.1451	2325.95***
	320	0.1504	2410.91***

Represented values are Mean  $\pm$  SD for 6 independent experiments. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001 statistically significant as compare with negative control

Implant material hydrophilicity is an important factor for successful implant osseointegration (Kumar *et al.*, 2017). Moreover, researcher demonstrates that hydrophilic implants have better prognosis than hydrophobic implants (Gittens *et al.*, 2014; Rupp *et al.*, 2011). Moreover surface coating with bone morphogenic protein, calcium hydroxyapatite and tricalcium phosphate have better osseointegration than conventional surfaces (Sartoretto *et al.*, 2015). This provides the knowledge that these synthetic products enhance osteoblast stimulation which favour bone deposition on the implant surface. Coating with easily available *Cissus quadrangularis* and lakshadi guugulu rather than bone mimetic coatings validate our present study. In this present study, aqueous extract of *cissus quadrangularis* and lakshadi guggulu which contain hydroxyapatite would be more promising agent in implant surface coating than methanolic extract.

In addition, increased alkaline phosphatase and lactate dehydrogenase activity is associated with bone regeneration and serves as a marker for bone regeneration (Rupp *et al.*, 2018). Ayurvedic products which enhance alkaline phosphatase and lactate dehydrogenase activity would be a promising factor in dental and orthopaedic implants which increase prominent osseointegration. However, surface coating of these extract on the titanium implants and the mode of delivery of these active ingredients at the site of fracture are still needed to identify. In the present study, methanolic extract of lakshadi guggulu possess better lactate dehydrogenase activity when compare to aqueous extract and *cissus quadrangularis* extracts, whereas the activity of alkaline phosphatase was higher in methanolic extract of *Cissus quadrangularis* and does not shows any significance with its aqueous extract.

## CONCLUSION

To conclude the present study, lakshadi guggulu has better effect over *Cissus quadrangularis* extract and possess osteogenic potential by increasing alkaline phosphatase and lactate dehydrogenase activity which further regenerates bone and can be used as a surface modification treatment for titanium implants. Further studies are in progress with these two products to validate the osteogenic potential.

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The authors declare that they have no funding support for this study.

## Conflict of Interest

The authors declare that there is no conflict of interest for this study.

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