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Original Research Article

Biochemical, radiological and histopathological assessment of bone regeneration properties of *Cissus quadrangularis*, a medicinal herb: A study on drill hole fractured bones of Wistar rats

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ABSTRACT

Background: *Cissus quadrangularis*, is commonly known as Hadjod in Hindi or bone setter due to its bone fracture healing properties. The presence of secondary metabolites plays an important role due to its characteristic properties. A number of studies were reported by the traditional healers for its bone healing properties but no clinical investigations including radiological and histopathological studies were done before in order for scientific validation of hadjod extracts.

Materials and Methods: The present study involves use of C-ARM X-ray under controlled condition for creating an artificial fracture of tibia bone of experimental Wistar rats. The phytochemical isolation was done by using successive extraction of plant material followed by chromatographic separation using polar to non-polar solvents. The radiological study was done using high resolution Helical CT scan by measuring variation in Hounsfield Unit (HU) value at proximal end of fracture at different time intervals. The histopathological study was done by the appropriate processing of slides at the fractured site. The histopathological changes were recorded and grading done on the basis of bone development stage within the callus.

Results: The histopathological results of one of the fraction (F-5) of petroleum ether extract showed mature bone formation grade - 9 on scale of bone healing which was higher than control group with grade - 5 on scale of bone healing. On the other hand crude ethanol extracts (A-4) showed the highest scale grade-10 of bone formation represents lamellar /mature bone formation.

Conclusion: Based upon radiological and histopathological studies, secondary metabolites presents in Petroleum ether and ethanol extract showed good results in terms of callus formation at fractured site.

1. Introduction

Bone fracture involves breaking of bone along with damage of soft tissues nearby area.[1] It is a fractional or total rupture in the connection of bone takes place when there is an contact of a high external force or impact on it.[2] It undergoes pain, swelling, edema, hematoma, collapse of periosteum, endosteum, and rupture of soft tissues.[3] There is time required to heal the fractured bone to restore the regular anatomical composition and avoid the chance of an irregular position.[4] In bone fracture

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therapeutics, reformation takes place from the fractured bone through three major mechanism inflammatory, repair, and remodeling stages.[5] The use of the laboratory synthesized compounds and minerals with Non-steroidal anti-inflammatory drugs (NSAID) on fractured bones areas resulted in poor fulfillment among the patient due to number of side-effects such as nephrotoxicity, inflammation, decline in blood flow, discomfort, pain, change in color of skin and nails, gastrointestinal blood loss, delayed blood clotting, and perseverance of treatment.[6] Therefore, there is a requirement of newer method of treatment to treat the fractured bone and to avoid such side effects. The literature study revealed that usage of natural source in terms of phytochemicals for bone healing treatment showed promising results without side effects and reducing repair period.[7] In this study, collected four fractions of *Cissus quadrangularis* (CQ) and their further fractions for the treatment of bone healing due to the wealthy content of calcium and other phytochemicals.

Cissus quadrangularis Linn.(Vitaceae)[8] has thick quadrangular-sectioned branches with internodes, fleshy herbs widely found in hotter zones of India and other warm tropical regions, locally named as Hadjod.

It has properties of accelerating fracture healing[9] and increasing bone strength and has been used as traditional /ethnic medicine by traditional healers. It has been used as a general tonic and analgesic especially for bone fracture healing.[10] It has 80-90 percent of success rate for callus formation ensuring early ossification and accelerating the phenomenon of fracture healing.[11] It is also called as the "Bone Setter" as it is the foundation of a phytogenic anabolic steroid used in bone healing.[12,13]

Cissus quadrangularis is well reported for their antibacterial, antioxidant[14,15] antifungal, hepatoprotective[16] anti-ulcer properties. Apart from this gastroprotective, anthelmintic, antihemorrhoidal, anxiolytic, antipyretic, antidiabetic,[17] antiviral,[18] analgesic, anti-inflammatory[19,20] and anti-glucocorticoid[21] properties are attributed to their bioactive constituents such as alkaloids, flavonoids flavones, triterpenoids, tannins and phenols. It also has a prominent activity against peptic ulcer disease.[22,23] Its extract has long been used as a natural remedy for the treatment of a wide variety of ailments and disorders.

2. Materials and Methods

2.1. Chemicals & reagents

All chemicals, solvents and reagents were purchased from Qualiens (India) Pvt. Ltd, Mumbai. Most common solvents like petroleum ether 60-80^{o}C, benzene, ethyl acetate, and ethanol were used in dry and distilled form. Alumina (make : Qualiens) of Grade II and silica gel 60-120 micron mesh size for column chromatography were used for all applications of separation of components. Thin layer chromatography was done on a glass plate by applying silica gel (200 micron) slurry through an applicator. TLC colour regents were prepared as prescribed standard methods.

2.2. Plant collection and experimental techniques

Herb *Cissus quadrangularis* Linn. (Vitaceae) was collected from the Herbal Garden Indira Gandhi Krishi Vishwvidyalaya. Dust free cleaned, shade dried and stem part of *Cissus quadrangularis* was pulverized in powdered form of 200-micron mesh size. Extracts were prepared using Soxhlet apparatus of 200 gram capacity. Successive extraction techniques were applied using a gradient system of solvent from non-polar to polar solvents. The process was done for 8-10 hr at temperature ranging between 60-80°C. Each extract was concentrated to dryness at 40°C in a rotary vacuum evaporator and stored at 4°C until when required for use.

All four extracts petroleum ether, benzene ethyl acetate, and ethanol were further fractionated by repeated column chromatography for isolation of molecules. The preliminary separation of each extract was done on activated alumina grade II (1:40 :: Extract : Alumina) as absorbent in stationary phase. Mobile phase of various solvents ranging from non-polar to polar solvents were used. Four fractions were collected by eluting petroleum ether, benzene, ethyl acetate, and ethanol. Further each fraction collected from alumina was separated by using activated silica gel on the glass column (1:30: Extract: silica gel). Three major fractions were collected by eluting solvents; petroleum ether, benzene, ethyl acetate, and ethanol. The result of each extract and its fractions was recorded on the TLC plate on the basis of Rf value using the gradient solvent system. Eluting solvent system and separation pattern of molecule were recorded. Based on TLC results, each fraction of silica gel was further separated over a silica gel column of small diameter. Number of fractions was collected by using a gradient solvent system. TLC was done of each fraction and the similar fractions having same Rf value on TLC were pooled. Out of four extracts, two extracts and their stepwise fractions of alumina column and then fractions of silica gel column are coded/abbreviations are as follows:

[A]: General Abbreviation: HCE: Hadjod crude Extract; Al: Alumina; Silica: Silica gel; PE: Petroleum ether; BZ: Benzene; EtoAc: Ethyl acetate; EtOH: Ethanol; CXX and HX-1: Control.

[B]: Extracts: Petroleum ether: A-1; Benzene: B-1; Ethyl acetate: C-1; Alcohol: D-1

[C]: Hadjod crude Petroleum ether extract:

F-5: HCE_PE/Al col^{n}_PE fraction

F-6: HCE_PE/Al col^{n}_BZ fraction

F-7: HCE_PE/Al col^{n}_EtoAc fraction

F-23: HCE_PE/Al col^{n} _PE/Silica col^{n} _ PE fraction

F-17: HCE_PE/Al col^{n} _PE/Silica col^{n} BZ fraction

F-18: HCE_PE/Al col^n_ PE/Silica col^n EtoAc fraction

F-20: HCE_PE/Al col^n_ EtoAc /Silica col^n_BZ

F-21: HCE_PE/Al col^n_ EtoAc /Silica col^n_EtoAc fraction

[D]: Hadjod crude Ethanol extract:

F-15: HCE_EtOH/Al col^n_BZ fraction

F-16: HCE_EtOH/Al col^n_EtoAc fraction

F-19: HCE_EtOH/Al col^n_BZ/Silica col^n BZ fraction

F-25: HCE_EtOH/Al col^n_BZ/Silica col^n EtoAc fraction

F-27: HCE_EtOH/Al col^n_EtoAc/Silica col^n BZ fraction

F-28: HCE_EtOH/Al col^n_EtoAc/Silica col^n EtoAc fraction

Initially the fractions of petroleum ether and alcohol extracts of hadjod were taken for this study. The both extracts has responded better than results as compared to benzene and ethyl acetate fractions. (Figure 1). TLC was done of each fraction and the similar fractions having same Rf value on TLC were pooled. After pooling the samples with same TLC picture, we collected 17 fractions of petroleum ether and alcohol fractions from hadjod extracts. The isolated fraction of each extract was applied on fractured areas of Wister rats to test radiologically and histopatholgically for their bone regeneration properties.

The fractions, which showed good response in terms of regeneration of callus tissues at fractured site, were considered as the active fraction having responsible components for bone healing present in that particular fraction.

The isolated fraction of each extract was applied on fractured areas of Wistar rats to record the callus formation in 21 days. Animal experiments were designed by using a pair of animals. The results of animal experiments were designated based on radiological (HU value at fracture end) and histopathological studies (histopathological grading of fracture healing). The fractions, which showed good response in terms of regeneration of callus tissues at fractured site, were considered as the active fraction having responsible components for bone healing present in that particular fraction. The active fractions were further separated by repeated chromatography over a silica gel column to fractionate the components using gradient system of elution of solvents.

The fractions, which showed good response in terms of regeneration of callus tissues at fractured site, were considered as the active fraction having responsible components for bone healing present in that particular fraction.

2.3. Animals experiment

Animal experiment on Wistar rats was conducted at the College of Veterinary Sciences and Animal Husbandry, Anjora, Durg to screen out the bone healing properties in fractured areas by applying extract externally. The proposed program was approved by the Institutional Animal Ethical Committee vide No. IAEC/VCA/Durg dated 03.02.2018, College of Veterinary Sciences and Animal Husbandry, Anjora, as per prescribed guidelines of the Committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Government of India.

IAEC has permitted 180 Wistar rats for the approved program. Each Wister rat having the weight of about 200-250g and age group of about 80-90 days were acclimatized in the small animal house at temperature 25±2∘C, controlled relative humidity conditions (50–55%) and 12 hr light-dark cycle. The animals were kept in the polypropylene cages and provided with standard rat feed and filtered water ad libitum.[24] The experimental animals were adapted for laboratory conditions 1 week before performing the study to evade apprehension in animals. All the experimental animals were housed under standard environmental conditions with temperature (23 ± 2 ^{o}C), humidity (50 ± 5%), and 12 h of light/dark cycles.[25]5. To ensure hygiene the rice husk in the cages was changed to new on every alternate day to provide maximum comfort for animals. All experimental animals were anesthetized by intramuscular injection of Ketamine (60-70 mg/g body weight + Diazepam 2-4 mg/g body weight). During the experiment, drilling was done under C-ARM X-ray by a highly sophisticated micro drill machine of caliber 1.5 mm at a defined length and position on the left tibia. Artificial fracture was created mechanically at the drilled site in each rat. Paste of each extract (500 μl /100 g body weight) was applied externally at the fractured area of Wister rat and proper bandaging was done to avoid infections. The albino rats undergo acute toxicity study as per OECD guidelines 42[26]30. Fresh extract was used for application on alternate days. Antibiotic and anti-inflammatory suspension was given orally. A spiral CT scan was recorded on 0, 7, 14, and 21 days at the fracture site. Health status of the animals was monitored during the treatment perio**d**.

2.4. Radiological study

Radiological examination, spiral CT (Computed Tomography) scan was performed at dept. of Radiodiagnosis, VY Hospital, Raipur, Chhattisgarh, India using an ACTS 16-slice resolution helical CT scanner (GE Healthcare, New York). The fractured ends of bone were studied for the test and control animal groups to evaluate the rate of healing. Radiographs of the fractuted bone were also taken throughout the observation period on 0, 7, 14, and 21 days. Hounsfield Unit (HU) value of the callus was studied at the fractured proximal end of bone. The Hounsfield units (HU) are standard numbers representing relative density of body tissues according to a calibrated gray-level scale, based on values for air (-1000 HU), water (0 HU), and bone density (+1000 HU).[27] The

percentage of variation in HU value was determined as follows: Percentage of Variation in HU value of test group = (HU value of 21 days /HU value of 0 day)*100. The obtained values were compared with the percentage of variation of control group i.e. = (% of variation of HU value of each extract – control value/control)*100.

2.5. Euthanasia and anesthesia

For the histopathological study, experimental animals were sacrificed by cervical dislocation as it causes extensive damage to the brainstem resulting in instant unconsciousness and death. Experimental rats were sedated with diethyl ether (5%) before dislocation.

2.6. Histopathological examination[28–30]

Histopathological examination of the fractured tibia was done by sacrificing one animal from each group. The histopathological samples were kept in 10% neutral buffered formalin. The specimens were decalcified with 5% nitric acid for 2-3 hours. After ensuring complete decalcification, four representative sections were taken from the fracture site and subjected to tissue processing. After processing, the tissues were embedded in paraffin and were sectioned to a thickness of 4μm using the rotary microtome. The sections were then stained by Harris hematoxylin and eosin stain. Microscopically the fracture site was evaluated on scanner view (4 x) and the histopathological changes were recorded for the relative proportion of necrosis with inflammation, fibrous tissue, cartilage, woven bone, and mature bone in the callus.

The progression of fracture healing was quantified with the use of a scale that assigns a grade based on the relative percentages of fibrous tissue, cartilages, woven bone, and mature bone in the callus. The grading is as follows.[29,30]

Grade 1: Necrosis with fibrous tissue

Grade 2: Predominantly fibrous tissue with some cartilage

Grade 3: Equal amounts of fibrous tissue and cartilage

Grade 4: All cartilage

Grade 5: Predominantly cartilage with some woven bone

Grade 6: Equal amounts of cartilage and woven bone

Grade 7: Predominantly woven bone with some cartilage

Grade 8: Entirely woven bone

Grade 9: Woven bone and some mature bone

Grade 10: Lamellar (mature) bone

2.7. Phytochemical studies

The extracts of *Cissus quadrangularis* (CQ), were subjected to phytochemical studies to get the active chemical compounds present in it. Extracts were sticky, green color, and stored at 4 °C until use. Phytochemical studies were performed as per the reported method prescribed for medicinal compounds using suitable chemical reagents. Extract have been reported to contain a large number of phytochemicals such as calcium, ascorbic acid, triterpenes, β-sitosterol, ketosteroid, asymmetrical, and tetracyclic triterpenoids.[31]

2.8. Drill-hole defect at the mid-diaphysis of the tibial

Adult albino rats (180 _ 20 g each) were taken and randomly divided into five groups for the drill-hole injury study. For this, the front skin of the mid- tibial in rats was incised straight and longitudinally at 1 cm in length under anesthesia. After splitting the muscle, we stripped the periosteum to expose the femoral bone surface. A drill-hole injury was made by inserting a drill bit with a diameter of 0.8 mm in the anterior portion of the diaphysis of the bilateral tibial 2 cm above the knee joint. Drill-hole injury in the tibial was created in order to do the uniform fracture.[32,33] Treatments started from the next day of injury and continued for 4 weeks. After 4 weeks of the various treatments described earlier such as radiological studies and day to day activities, later on all rats were euthanized and autopsied to collect their tibial for the measurement of bone for dynamic histomorphometric study at the fractured site.[34]

3. Result and Discussion

3.1. Phytochemical results

As mentioned in above section, the preparation of extract, successive extraction techniques were applied using a gradient system of non-polar to polar solvents. Four main core extracts namely Petroleum ether, Benzene, Ethyl acetate and Ethanol were collected.

Phytochemical studies on extracts of *Cissus quadrangularis* (CQ) revealed that the presence of numerous bioactive compounds such as flavonoids, calcium, vitamins, alkaloids, resveratrol, ascorbic acid, phytosterol, triterpenoids, nicotinic acid, carotene, enzyme, quadrangularins, pallidol, parthenocissin, kaempferol, phosphorus, tannins, glycoside, saponins, carbohydrates, anthraquinones, protein, fatty acid, moisture, and ash.[31] Every extracts is different from the others by appearances, metabolites present in it, and their yield. Extractive values of different extracts of *Cissus quadrangularis* in different solvents are demonstrated in Table 1.

3.2. Radiological & histopathological study

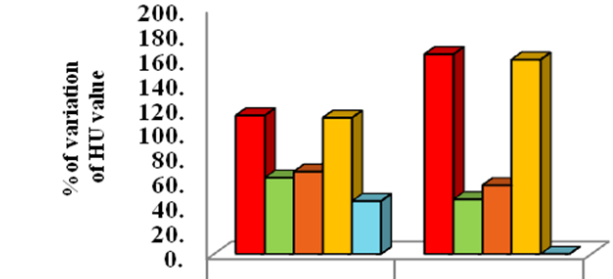
The Petroleum ether, Benzene, Ethyl acetate and Ethanol crude extracts were tested to find out the efficient fraction which has the capacity for more callus formation /bone regeneration properties. Out of the crude extracts, the one which shows better bone healing response were proceeded by further fractionation using various adsorbents in chromatographic techniques to get the final fraction contains responsible secondary active molecules.

Table 1: Successive solvent extraction of *Cissus quadrangularis* (Hadjod powder)

Plant Name/Part Used	Quantity	Time Duration of Extraction	Method of Extraction	Solvents Used	Color	% Yield
Cissus quadrangularis/ Stem	200 g	8 hrs for each fraction	Continuous hot Percolation by Soxhlet Apparatus	Pet. Ether	Light Green	4.0%
				Benzene	Olive Green	1.5%
				Ethyl Acetate	Green	1.6%
				Chloroform	Dark Green	1.8%
				Water	Brown	3.2%

In the first step the four crude extracts viz. petroleum ether [A-1], benzene [B-1], ethyl acetate[C-1] and ethanol [D-1] taken for Radiological & Histopathological study to see bone healing properties at proximal end of fractured end of bone.

Radiological studies was recorded for the determination of percentage of variation in HU value of CT scan of four crude extracts i.e petroleum ether [A-1], benzene [B-1], ethyl acetate [C-1] and ethanol [D-1] was found as 162.15; 44.42; 55.76 and 157.78 respectively (Figure 1). The percentage of variation in HU value of CT scan of Petroleum ether [A-1] 112.46 and Ethanol extracts [D-1] was 110.58, recorded higher than control 42.91[XF-1].



	% of variation	% of variation> control
A-1 Petroleum ether	112.46	162.15
B-1 Benzene	61.96	44.42
C-1 Ethyl acetate	66.82	55.76
D-1 Alcohol	110.59	157.78
XF-1 Control	42.91	0.00

Figure 1: Comparative studies of percent of variation in HU value of CT scan & variation percent increased with control of Crude fractions: Petroleum ether : Benzene: Ethyl acetate and Alcohol extracts of Hadjod

The histopathological studies revealed that extracts A-1 and D-1 showed grade-7 and grade–10 bone formation as per bone healing staging i.e. formation of predominantly woven bone with some cartilage and Lamellar/mature bone respectively as comparable to control which showed grade-3 staging (Equal amounts of fibrous tissue and cartilage). B-1 and D-1 showed grade-2 bone formation as per the staging that indicates predominantly fibrous tissue with some cartilage and was lower grade than control (Figure 2).

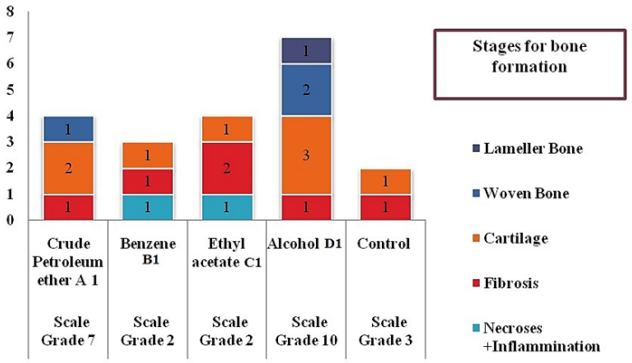


Figure 2: Histopathological observation and grade of petroleum ether, benzene, ethyl acetate and alcohol crude extract

The CT scan and histopathological indicates that out of above four extracts only petroleum ether and ethanol extracts showed good response for callus formation/bone healing properties reflecting the presence of secondary metabolites responsible for bone healing. Hence, these two extracts petroleum ether and alcohol extracts were taken for further study.

The crude petroleum ether A-1 was further under chromatographically fractionation to get seven more fractions viz. F-5, F-23, F-17, F-18, F-7, F-20 and F-21 which were screened radiologically and histopahtologically to find out the best fraction showed good response in bone regeneration capabilities. The percentage variation in HU value and grading of various stages of bone formation is showed in Figures 3 and 4.

The histopathological results of one of the fraction (F-5) of petroleum ether crude extract showed mature bone formation grade - 9 on scale of bone healing (woven bone and some mature bone formation) which was higher than control group which showed grade - 5 on scale of bone healing (predominantly cartilage with some woven bone). The other fractions F-23, F-17, F-18, F-7, F-20 and F-21 of crude petroleum extract (A-1) showed grade-5, 5, 4, 6, 6, and 3 respectively on scale of bone healing as compared to the control group with grade-5. Histopathlogical images of

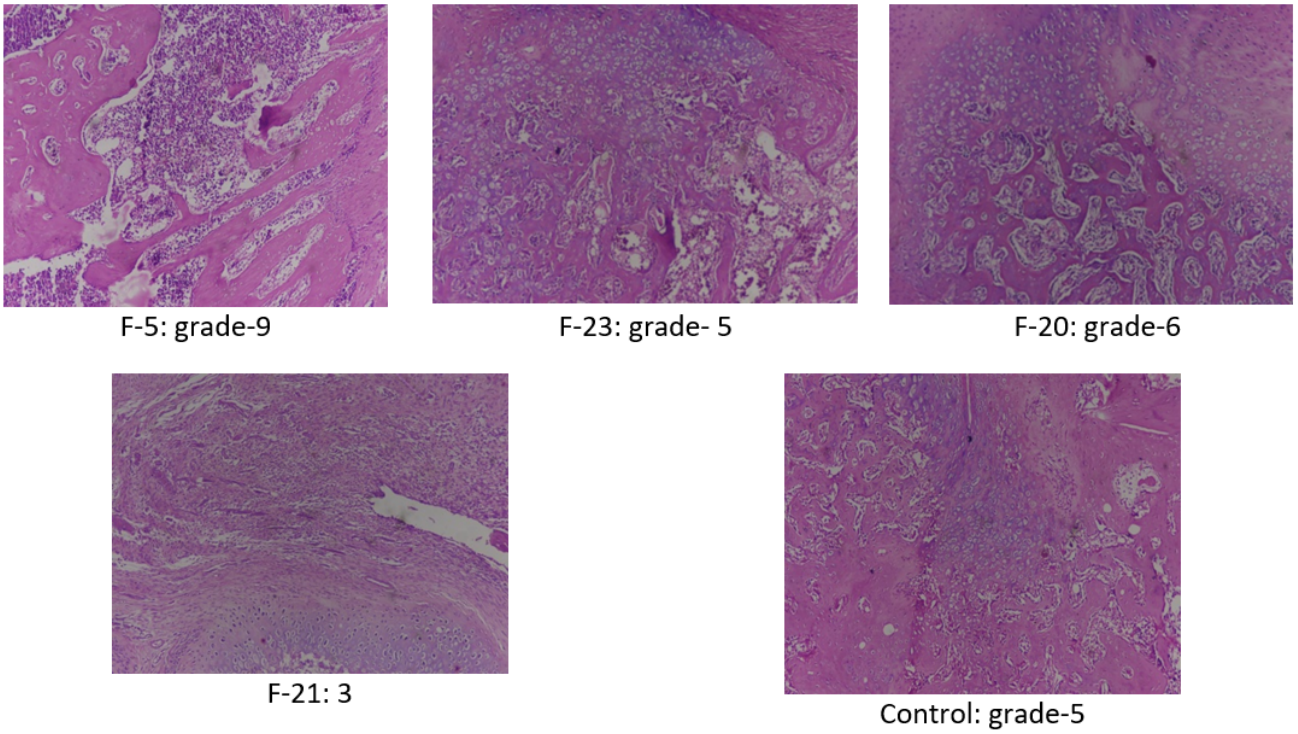
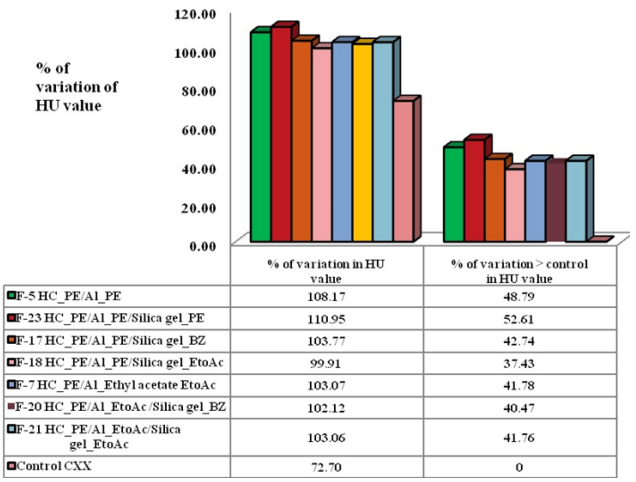


Figure 5: Histopathological images of various fractions of petroleum ether extracts



	% of variation in HU value	% of variation > control in HU value
F-5 HC_PE/A1_PE	108.17	48.79
F-23 HC_PE/A1_PE/Silica gel_PE	110.95	52.61
F-17 HC_PE/A1_PE/Silica gel_BZ	103.77	42.74
F-18 HC_PE/A1_PE/Silica gel_EtoAc	99.91	37.43
F-7 HC_PE/A1_Ethyl acetate EtoAc	103.07	41.78
F-20 HC_PE/A1_EtoAc/Silica gel_BZ	102.12	40.47
F-21 HC_PE/A1_EtoAc/Silica gel_EtoAc	103.06	41.76
Control CXX	72.70	0

Figure 3: Comparative studies of percent of variation in HU value of CT scan & variation percent increased with control of fractions of Petroleum ether extracts

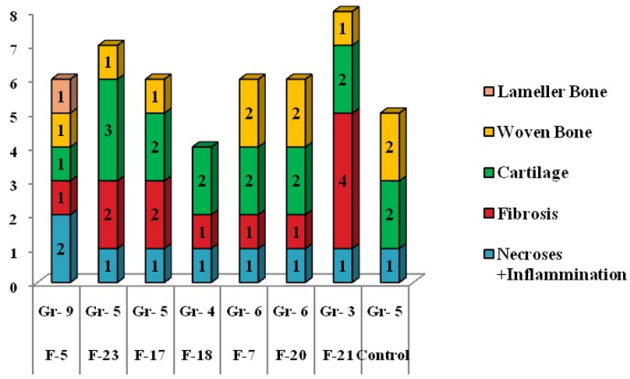


Figure 4: Histopathological observation and grade of various fractions of petroleum ether extracts

few fractions are shown in Figure 5.

The crude ethanol D-1 was further under chromatographically fractionation to get seven more fractions viz. F-15, F-19, F-25, F-16, F-27, and F-28 which were screened radiologically and histopahtologically to find out the best fraction showed good response in bone regeneration capabilities. The percentage variation in HU value and grading of various stages of bone formation is showed in Figures 6 and 7.

Histopathological results indicate the crude ethanol extracts (A-4) showed the highest scale grade-10 of bone formation represents lamellar /mature bone formation. The histopathology observations of one fraction (F-27) revealed predominantly woven bone with some cartilage bone (Grade-7) while another fraction (F-28) fraction showed woven bone and some mature bone (Grade-9). Both of these fractions showed good response for bone healing. These fractions showed 3-5 spots on TLC plates gives the

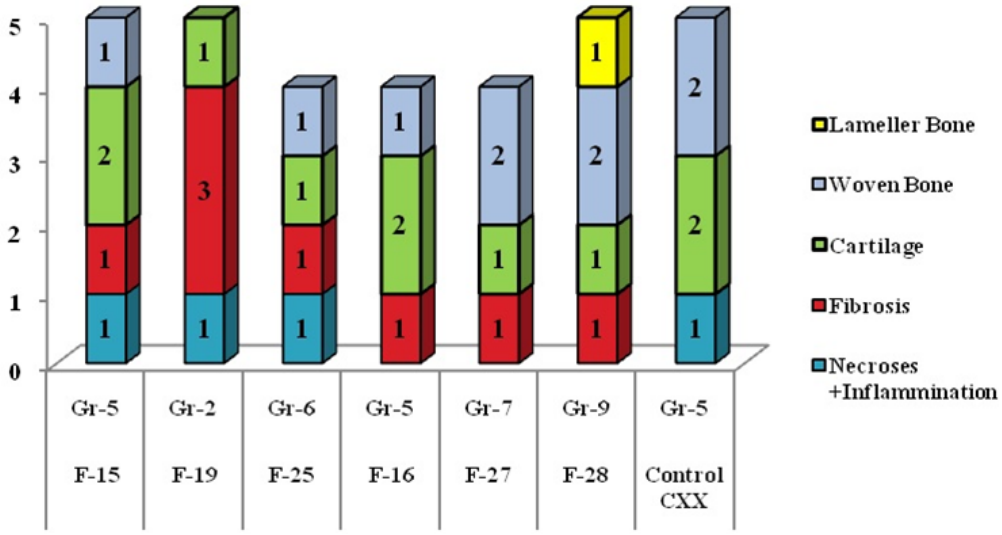


Figure 7: Histopathological observation and grade of various fractions of petroleum ether extracts

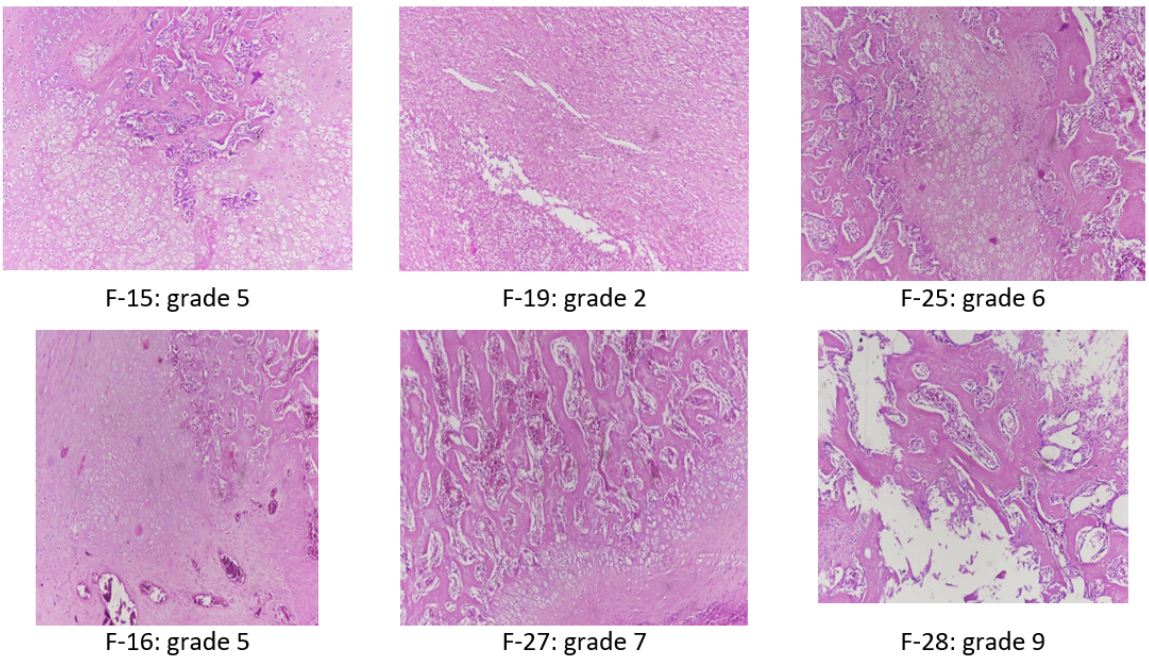
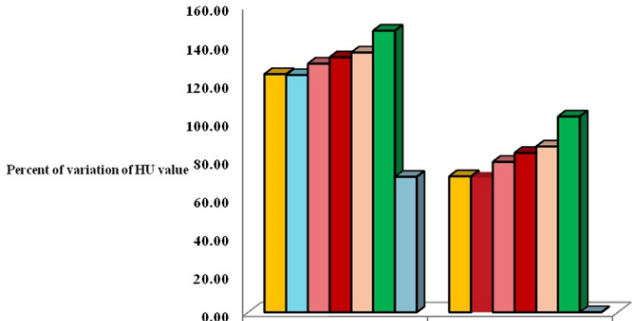


Figure 8: Histopathological images of various fractions of petroleum ether extracts

probability of 3-5 secondary metabolites responsible for bone healing characteristics in the herb.

The histopathological results of the fractions F-27 and F-28 of alcohol crude extract which was higher than control group which showed grade - 5 on scale of bone healing (predominantly cartilage with some woven bone). The other fractions F-15, F-19, F-25, and F-16 of crude alcohol extract (D-1) showed grade-5, 2, 6, and 5 respectively on scale of bone healing as compared to the control group with grade-5. Histopathlogical images of few fractions are shown in Figure 8.

In summary based on radiological and histopathological studies out of crude four extracts only two extracts namely petroleum ether (A-1) and ethanol (D-1) showed best performances of bone regeneration activity. Among crude petroleum ether extract (A-1), the fraction F-5 showed good response while the in case of crude ethanol two fractions viz F-27 and F-28 showed the excellent response on callus formation based on radiological and histopathological data.



	% of variation in HU value	% of variation > control in HU value
F-15 HC_EtOH/Al_BZ	124.30	70.98
F-19 HC_EtOH/Al_BZ/Silica gel_BZ	123.81	70.30
F-25 HC_EtOH/Al_BZ/Silica gel_EtoAc	129.77	78.50
F-16 HC_EtOH/Al_EtoAc	133.15	83.15
F-27 HC_EtOH/Al/EtoAc/silicagel_BZ	135.62	86.55
F-28 HC_EtOH/Al/EtoAc/silicagel_Eto Ac	147.00	102.20
CXX Control	70.72	0.00

Figure 6: Comparative studies of percent of variation in HU value of CT scan & variation percent increased with control of fractions of Petroleum ether extracts

As per the radiological and histopathology results, the fractions of petroleum ether and ethanol fraction has good response of callus /bone formation capacity of callus regeneration in proximal end of fractured tibia bone of experimental animal. It means the presence of secondary metabolites in these fractions which played an important role in bone formation. The nature of the molecules and their isomers may be aliphatic; aromatic cyclic in nature having high molecular weight may be present which will be isolated and characterized in due course.

4. Source of Funding

None.

5. Conflict of Interest

Authors declare no conflicts of interest.

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