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IN VITRO ANTIMICROBIAL ACTIVITY OF OLEANOLIC ACID IDENTIFIED IN CHLOROFORM EXTRACT OF FRUITS OF *HELICTERES ISORA* L.

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ABSTRACT

The present study investigates the isolation and characterisation of Oleanolic acids from chloroform extract of fruits of *Helicteres isora* Linn and aim to prove Oleanolic acid as a antimicrobial agents that can be develop a new chemotherapy as antibiotics. *Helicteres isora* L is belongs to family Sterculiacae is a sub-deciduous shrub or small tree. It occurs, throughout India. The fruits are ground and used in colic pain, gripping bowels and flatulence. The fruits are astringent, acrid, refrigerant, demulcent, constipating, stomachic, vulnerary, haemostatic and urinary astringent. After chromatographic separation of compound HFCA, Antimicrobial activity have been performed and then chemical examination of HFCA done by IR,NMR techniques and finally it is proved that HFCA as a Oleanolic acid and zone of inhibition against *S.aureus, E.coli, P.aeruginosa, B.cereus* and *A.flavus* determines that Oleanolic acid acts as antimicrobial agents.

KEYWORD: *Helicteres isora* L, Antimicrobial activity. IR/UV,NMR Oleanolic acids.

INTRODUCTION

Helicteres isora L is belongs to family Sterculiacae, commonly known as East Indian screw tree, is a medicinally important, sub-deciduous shrub or small tree of reaching a height of 5-15 feet. Almost all parts of the plant are used in traditional medicinal system for curing

various diseases.[1-3] Water extract of the fruits exhibits anti-HIV activity[4] and reported to possess antispasmodic activity.[5] The roots showed significant hypolipidaemic and hypoglycaemic[6-8], while bark has shown significant hypoglycaemic activity.[2] The species is native to Asia and Australia. It occurs throughout India.The fruits are ground and used in colic pain, gripping bowels and flatulence. The fruits are astringent, acrid, refrigerant, demulcent, constipating, stomachic, vermifuge, vulnerary, haemostatic and urinary astringent. They are useful in colic, flatulence, diarrhoea, dysentery, verminosis, wounds, ulcers, hemorrhages, epistaxis and diabetes. The root and stem barks are considered to be expectorant, demulcent, astringent and anti-galactagogue and are useful in colic, scabies, empyema, gastropathy, diabetes, diarrhoea and dysentery. The fruits were also found to possess significant antispasmodic activity In the present investigation, Chloroform extract were obtained by using soxhlet extraction method from the fruits of *Helicters isora* Linn and Initial results obtained from thin layer chromatography (TLC) showed the good pattern. Further IR, NMR analysis exhibited the structure of Oleanolic acid. Extracted Oleanolic acid was further used to determine its antimicrobial activity by agar disk diffusion method. The antimicrobial activity ranged from 10 mm to 17 mm for *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *B.cereus*. In comparison with standard antibacterial compound Tetracycline (20µg/ml), it was found that purified Oleanolic acid has antimicrobial activity.

MATERIALS AND METHODS

Plant collection and identification

Shade dried fruits of *Helicteres isora* Linn. were obtained from the Himalaya Drug company, Dehradun, Uttarakhand. The specimen was identified and confirmed by the Botanical Survey of India, Dehradun, India.

Experimental Procedure

The solvents and chemicals used for this work are laboratory grade petroleum ether, chloroform, methanol, acetic anhydride, ferric chloride, glacial acetic acid and sulphuric acid. Column chromatography (CC) was conducted on silica gel 60 (Kieselgel 100-200 µm particle size, E. Merck, Darmstadt, Germany) and TLC was performed on silica gel 60 F_{254} precoated aluminium plates (layer thickness 0.2 mm, E. Merck, Darmstadt, Germany). IR spectra were measured on a Shimadzu FTIR DRX-500 in KBr pellets. ^{1}H NMR (400 MHz), ^{13}C NMR (150 MHz) and 2D NMR spectra were recorded on Bruker Ultrashield 400 spectrophotometer with TMS as the internal standard.

Preparation and Extraction of Plant material

The fruits (2.0 kg) of *H. isora* were shade dried at room temperature, chopped into small pieces was exhaustively defatted with petroleum ether (60-80°C) and successively extracted with chloroform ($CHCl_3$) and then finally methanol (MeOH) using the soxhlet apparatus for 6 hours. Removal of the solvents under reduced pressure gave the respective extracts. The yield of the extracts was determined to be 12 gm, 22 gm and 55 gm, respectively. TLC examination of $CHCl_3$ extracts showed good patterns. Hence these extracts are used for chemical examination. The MeOH; 50 gm extracts was suspended in water and sequentially extracted with ethyl acetate (10 gm) and n-butanol (35 gm). These extracts were concentrated in vacuum and stored at 4°C until examined.

Fig-1-Prepared plant material of *H. isora* fruit parts for analysis

Examination of Chloroform Extract

Column chromatography of the chloroform extract (11.0 g) was carried out on silica gel. The column was eluted with increasing amount of ethyl acetate (EtOAc)/methanol in chloroform. Different fractions of 50 mL each were collected, examined on TLC and combined based on their TLC behavior. The column on elution with $CHCl_3$-methanol (96:4, v/v) afforded one pure compounds HFCA.

Compound HFCA

The fractions eluted with $CHCl_3$-methanol (97:3, v/v)gave on concentration yielded a white powder which was crystallized with methanol as white needles (21 mg), m.p. 299-301°C. It gave positive Libermann Burchard test indicating triterpenoid nature of the molecule.

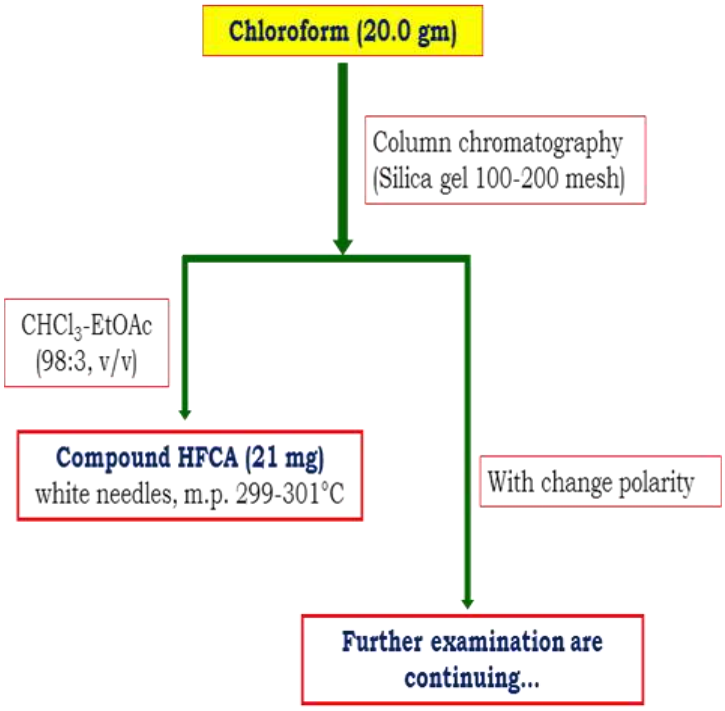


Fig-2-COLUMN CHROMATOGRAPHY: HFCA

Fig-3-TLC PATTERN: HFCA

HPLC-UV Spectroscopy

λ_{max} $CHCl_3$nm: 231.

IR Spectroscopy

IR (cm^{-1}): ν_{max}KBr,3512, 3367, 2926, 2870, 1687, 1516, 1458, 1386, 1377, 1269, 1238, 1188, 1089, 1030, 997, 856 (Figure 1).

^{1}H NMR Spectroscopy (500 MHz, $CDCl_3$)

δ (ppm): 5.27 (1H, t, *J* = 4.0 Hz, H-12), 4.50 (1H, t, *J*= 5.2 Hz, H-3), 0.83, 0.85, 0.88, 0.91, 0.93, 0.95, (each 3H s), 1.21 (3H, s), 2.38 (2H,dd, *J*=4.0 and 8.0 Hz), 2.75 (2H, dd, *J*=2.0 and 11.6 Hz), 1.90 (2H, m), 0.95-1.78(m) (Figure 2-4).

^{13}C NMR Spectroscopy(100 MHz, $CDCl_3$)

δ(ppm): 38.27 (t, C-l), 23.05 (t, C-2), 80.6 (d, C-3),37.59 (s, C-4), 55.4 (d, C-5), 18.14 (t, C-6), 31.72 (t, C-7), 40.04 (s, C-8), 47.6 (d, C-9), 36.70 (s, C-10), 22.02 (t, C-11), 122.56 (d, C-12), 143.78 (s, C-11), 41.99 (s, C-14), 27.70 (t, C-15), 23.45 (t, C-16), 46.6 (s, C-17), 40.26 (d, C-18), 45.54 (t, C-19),30.12 (s, C-20), 33.90 (t, C-21), 32.52 (t, C-22), 28.08 (q, C-23), 16.45 (q, C-24), 15.36 (q, C-25), 17.16 (q, C-26), 25.84 (q, C-27), 183.79 (s, C-28), 33.02 (q, C-29), 24.04 (q, C-30) (Figure 5).

EI MS (m/z)

456 [M^+], 441, 430, 411, 400, 391, 329, 325, 307, 289, 271, 248, 207,203, 189, 133, 107.

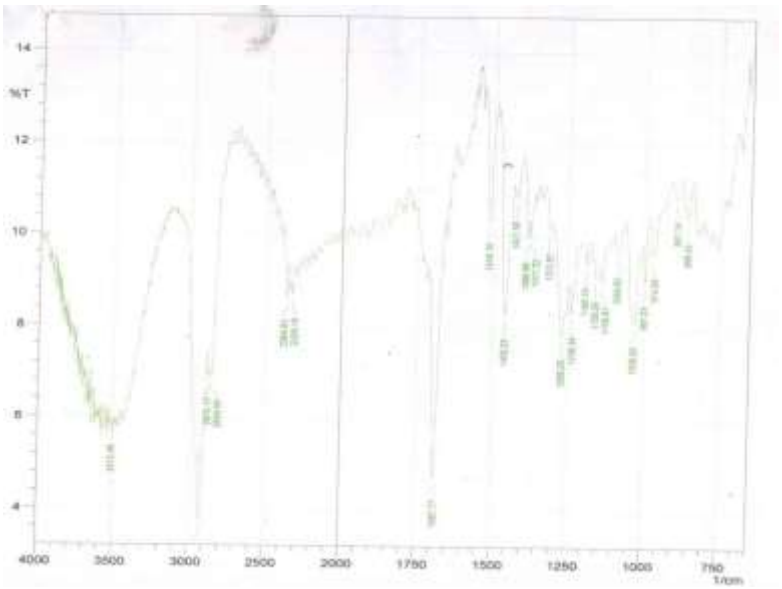


Fig:4- IR spectra of compound HFCA

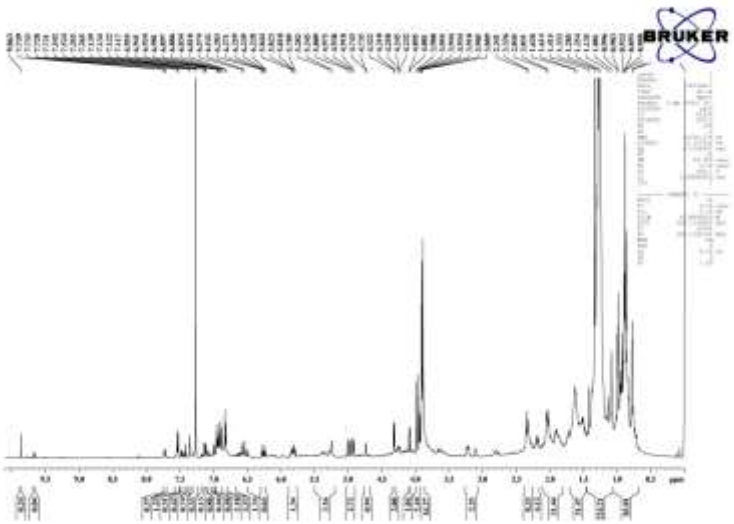


Fig: 5- ^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of HFCA

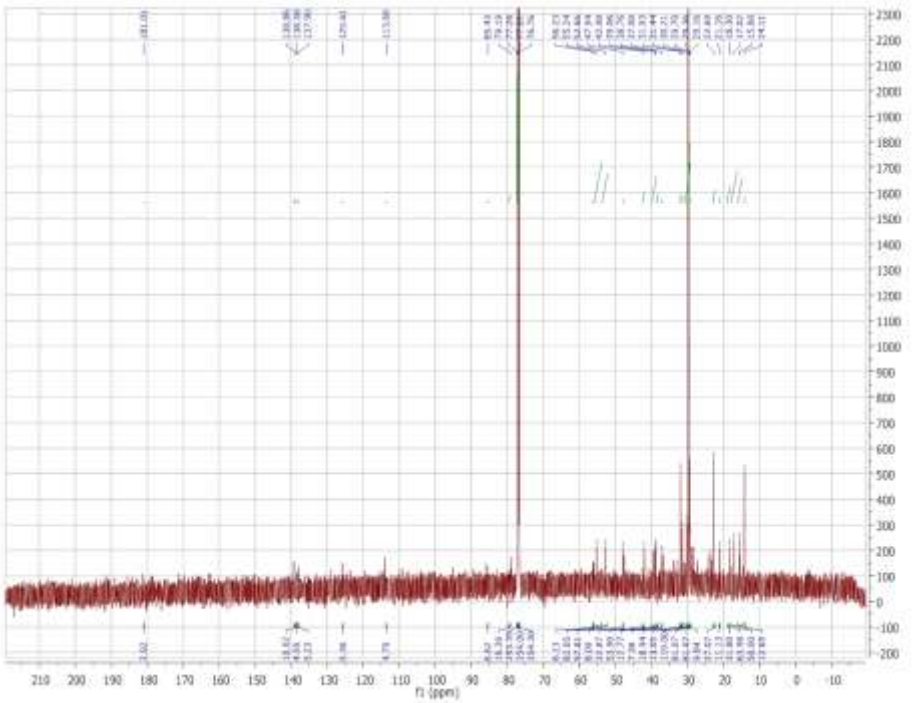


Fig-6-^{13}C-NMR (150 MHz, $CDCl_3$) spectrum of HFCA

ANTIMICROIAL PROPERTIES OF OLEANOLIC ACID (HFCA)

The antimicrobial properties of HFCA were assased against different bacterial and fungal species and the obtained results suggested the importance of this compounds as antibiotic drugs. One of the first studies that aimed to evaluate the possible effect of HFCA against bacteria was developed by Kozai et al.[9] In this work, it was demonstrated that this triterpenes inhibited the synthesis of insoluble glucan, catalyzed by crude glucosyltransferase

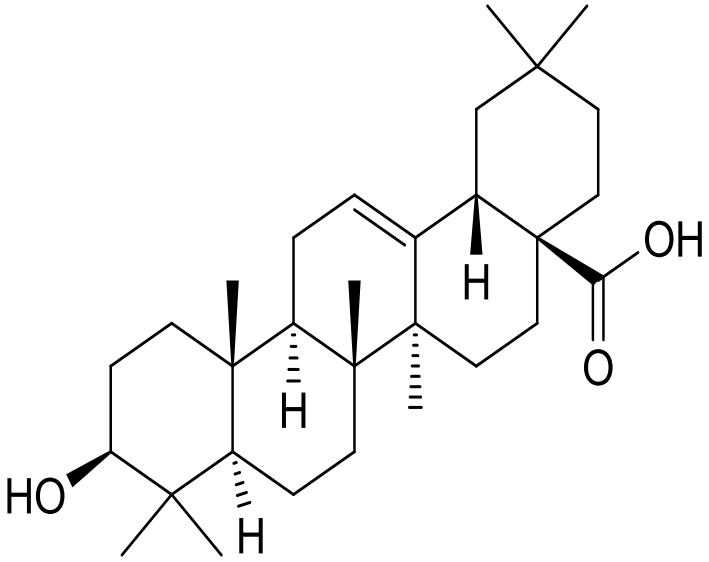
(GTase) from cariogenic *Streptococcus mutans*. When used against *Mycobacterium tuberculosis*, which is a bacterium that affects around one-third of the human population and represents the infection that causes the most deaths worldwide, it was found that HFCA isolated from *Lantana hispida* was also effective at displaying a MICvalue of 25 μg/mL.[10] In addition, a MIC of 50 μg/mL was reported when HFCA was used against *M. tuberculosis* streptomycin-, isoniazid-, rifampin- and ethambutol-resistant strains. Similar to HFCA purified from *Chamaedorea tepejilote* leaves was capable of eliminating *M. tuberculosis* at 100 μg/mL[11], suggesting that there is a potential for HFCA compounds to kill this pathogen. The diversity of the antibacterial properties of HFCA also been illustrated against other human bacterial pathogens, such as *S. pneumonia* (MIC of 16 μg/mL), methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* (MIC of 8 μg/mL and 64 μg/mL, resp.)[12], *Bacillus subtilis* (MIC of 8 μg/mL), *B. cereus*, *Enterococcus faecalis* (MIC of 6.25–8.00 μg/mL), *E. faecium*(MIC of 8 μg/mL), and *Pseudomonas aeruginosa* (MIC of 256 μg/mL).[13–15]

RESULTS

COMPOUND HFCA

It was obtained by elution of the column with chloroform and methanol (96:4, v/v), and crystallized with methanol crystallized from MeOH as white needles (21 mg), m.p. 299-301°C. It gave positive Libermann-Burchard test indicative the presence of triterpene skeleton. The IR spectrum of the compound exhibited characteristic absorption bands for -OH function at 3512cm^{-1}, for C-H stretching at 2926 and 2870 cm^{-1}, for carboxylic function at 1687 cm^{-1}, 1458 cm^{-1}. The UV spectrum of the compound showed characteristic maxima at 231 nm. The mass spectrum of the compound indicated its molecular weight 456 corresponding to the molecular formula $C_{30}H_{48}O_3$. The 'H-NMR spectrum of compound HFCA, exhibited the presence of seven methyl groups at δ 0.83, 0.85, 0.88, 0.91, 0.93, 0.95, and 1.21 and a characteristic olefinic proton of C_{12}-C_{13} double bonded pentacyclic-triterpenoid at δ 5.27 (1H, *t, J* = 4.0 Hz, H-12). DEPT spectrum of the compound displayed presence ofseven methyl, ten methylene, five methine and eight quaternary carbon atoms in the molecule. The ^{13}C-NMR spectrum of the compound revealed presence of signals due to an oxygenated carbon atom at δ 80.6 (C-3), one tri-substituted double bond at δ 122.56 (C-12) and 143.78 (C-13) and one carboxyl group at 184.79 (C-28). The ^{13}C-NMR signals due to C-18-C-22 [40.26 (C-18), 45.54 (C-19), 30.12 (C-20),33.90 (C-21), and 32.52 (C-22) suggested that Compound HFCA was an olean-12-en derivative. HFCA (3β-hydroxyolean-

12-en-28-oic acid) is a pentacyclic triterpenoid with widespread occurrence throughout the plant kingdom. HFCA was previously isolated from almost 2000 plant species[16–18] and the main source of this compound includes plants belonging to the Oleaceae family, such as *Olea europaea* (the olive).[19,20] In plants, the biological roles of this compound are often associated with the formation of a barrier against water loss and pathogens.[21] Moreover, allelopathic properties have already been described for this compound.[22] Several medicinal plants produce and accumulate HFCA and its derivatives as their main metabolites, which could be directly associated with their biological activities.



OLEANOLIC ACID

ANTIMICROBIAL ACTIVITY OF OLEANOLIC ACID

In the present study, the antimicrobial activity of the purified Oleanolic acid was evaluated and the results are summarized in the Table-1.

Table: 1 - Antimicrobial activity of Oleanolic acid

	Diameter of Zone of inhibition	
Test organism	**Oleanolic acid**	**Tetracycline**
1 *E.Coli*	14mm	18 mm
2. *Staphylococcus aureus*	10 mm	18 mm
1. *Pseudomonas aeruginosa*	17 mm	20 mm
4. *Bacillus cereus*	12 mm	16 mm
5. *Staphylococcus mutans*	18 *mm*	21 *mm*
5. *Aspergillus flavus*	11 mm	15 mm

The antimicrobial activity, determined by measuring the Zone of inhibition around the disc, ranged from 10 mm to 18 mm for the Oleanolic acid. Tetracycline showed the inhibition zone for *E. coli* (18 mm), *S. aureus* (18 mm), *P. aeruginosa* (20 mm), *Bacillus cereus* (16 mm), *Staphylococcus mutans*(21 mm), *Aspergillus flavus*(15 mm), while Oleanolic acid recorded 14 mm (*E. coli*), 10 mm (*S. aureus)*, 17 mm (*P. aeruginosa*), 12 mm (*Bacillus cereus*),18 mm(*Staphylococcus mutans*), 11 mm(*Aspergillus flavus*). Thus, the antimicrobial activity of the Oleanolic acid (10mm to 14mm) was comparable to the standard Tetracycline (18 mm to 16 mm). Hence Oleanolic acid is having higher antimicrobial activity against *S. Mutans* followed by *P. Aeruginosa, B.cereus* and *S. aureus* at lower concentration. Thus these results prove the antimicrobial potential of Oleanolic acid from the *Helicteres isora* and further, it can serve as an alternative chemical to treat the bacterial and fungal diseases.

CONCLUSION

Our studies reveals that Oleanolic acid shows remarkable antimicrobial activities against important human pathogens which is identified in chloroform extract of fruits of Helicteres isora Linn. In addition, these types of strategies will be crucial in the development of new drugs that can be used for populations that are at risk for contracting certain diseases. Our study contributes to establish Oleanolic acid as potent antimicrobial agent at lower concentration against a wide range of bacteria. However, still more scientific evaluation and clinical trials are required to establish its therapeutic efficacy.

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