

Original Research

Ursolic acid from leaf extracts of *Barleria lupulina* acting as anti clastogenic and anti tumor agent.

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ABSTRACT:

In our previous study, we have reported the radio-protective, anti clastogenic and anti tumor activities of the leaf extract of *Barleria lupulina* on mice and fish. In the present work, biological active component from the leaf extract has been isolated, by column chromatography. Phytochemical analysis, TLC and NMR studies confirm the presence of ursolic acid as the main anti cancer component, besides other such components, as β sitosterol, sitosterol-3-O-glucoside are also present.

Keywords:

Barleria lupulina; Acanthaceae family; anti-cancer, anti-tumor activities; ursolic acid; β sito sterol; sitosterol-3-O-glucoside.

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INTRODUCTION

Tumor formation and cancer is a life threatening disease and a problem to mankind all over the world. Tumors in malignant form lead to cancer. Search for anti cancer and radio protective agents from plant extracts is aimed by researchers all over the globe. Since the discovery of Patt *et al.*, (1949) that cysteine protects against X-irradiation in rats and mice, several workers are engaged in active research for the search of radio-protective component, even from plant extracts. Jagetia *et al.*, (2003) proved that naringin, a citrus flavonone protects mice from γ -irradiated chromosomal damage. Kanchanapoom *et al.*, (2001) reported that *Barleria lupulina* plant is externally used as an anti-inflammatory agent. Its anti diabetic potential has been stated by Suba *et al.*, (2004). Kanchanapoom *et al.*, (2001) isolated iridoid glucosides from the aerial parts of *Barleria lupulina*.

In our previous experiments (Sur and Das, 2012), we reported for the first time the anti cancer, anti clastogenic, radio-protective properties of the leaf extract of *Barleria lupulina* Lindl; on laboratory models as mice and anti tumor property on fish. Investigation of active component in the leaf extract is carried out in this study. Ursolic acid has been found to be the main component in the leaf extract, which pertains to its anti cancer property.

MATERIALS AND METHODS

Plant Material

Barleria lupulina of Acanthaceae family (common name hophead Philippine violet), was identified from the Central National Herbarium, Botanical Survey of India, Howrah-711103 (Ministry of Environment and Forests, Govt. of India). Leaves of this plant (Fig 1) were cleaned, washed and dried in shade. After sufficient drying, they were subjected to Soxhlet extraction in ethanol and its leaf extract (LE) was obtained as described in our previous study

(Sur and Das, 2012).

Instrumentation

NMR (Bruker Corp.) was operated at 400 MHz. Data were recorded by dissolving 15 mg of sample in 1 ml deuterated chloroform (CDCl_3). Column chromatography was operated using a glass column of 1 meter length, and 10 cm diameter packed with silica gel C (mesh size 100-200, EMERCK). Column was packed following wet packing method, with 100% petroleum ether. Solvent systems used for elution were petroleum ether (non-polar solvent), chloroform, ethyl acetate (medium polar solvents), methanol and ethanol (highly polar solvents) according to increasing order of their polarity. Elution was started with 100% petroleum ether (isocratic non-polar solvent), then the concentration of petroleum ether was gradually decreased and that of chloroform was gradually increased (gradient eluent). Then 100% chloroform was used (isocratic eluent). After this, medium polar solvent as ethyl acetate was used along with chloroform, for elution (gradient eluent). This was followed by 100% ethyl acetate (isocratic eluent, medium polar solvent). Methanol (a more polar solvent) was used next. The concentration of methanol was gradually increased and ethyl acetate was decreased. Gradually 100% methanol was used. And finally ethanol (most polar solvent) was used to washing off the column.



Fig (i): Leaves of *Barleria lupulina*

Table 1 Standards and mobile phase used in TLC to identify the active component by trial and error method

Sl No	Phytochemical Results	Standards Used	Mobile phase used	Ratio of mobile phase
1	Terpenoid	Ursolic acid	Methanol : chloroform	7:43
2	Steroid	β Sitosterol	Benzene : petroleum ether: chloroform: ethyl acetate	4:2:3:1
3	Glycoside	Sitosterol-3-O-glucoside	Benzene : chloroform: ethyl acetate: methanol	18:2:1:4

Extraction and Isolation by column chromatography

After Soxhlation 1.75 gm of LE was dissolved in adequate amount of 99.9% ethanol. Then it was mixed with silica gel C, and dried to remove the solvent. This was further used for column packing. Column was packed with silica gel C mixed in 100% petroleum ether by following wet packing method. Elution was done by using varying concentrations of non-polar, medium polar and polar solvents such as petroleum ether, chloroform, ethyl acetate, methanol and ethanol according to their increasing order of polarity.

After elution, each fraction was heated on water bath to evaporate the solvents and concentrate their constituents. Qualitative analysis of the phyto-constituents present in the fractions was performed by standard phytochemical tests described in Trease and Evans (1997).

Preparative TLC (for identification of active component)

Preparative TLC was performed by using standard 60F₂₅₄ TLC plates (pore size; 60 Å, uv fluorescence at 254 nm, Merck, Germany). The silica gel which acted as the stationary phase and mobile phase was chosen by trial and error method. Very small amount of the fractions from column chromatography (test) were put on the plates with the help of a capillary tube along with the standard phyto-constituents; and TLC was run using the mobile phase. Components of the fractions were identified by comparing with R_f value of the standards. Different standards and mobile phases were used for the comparison study. Standards and mobile

phase that showed fruitful results are summed up in (Table 1).

¹H NMR study of the fractions

¹H NMR of the fractions was performed by dissolving 15 mg of each fraction in 1 ml (CDCl₃). The NMR machine (Bruker Corp.) was operated at 400 MHz.

RESULTS**Column Chromatography and Phytoconstituents Present (Qualitative)**

A total of 52 fractions were obtained from column chromatography. The use of non-polar, medium polar and polar solvents for elution is mentioned in materials and methods. Compounds in pure form as terpenoids were isolated in fractions 2-5 (eluted with petroleum ether and chloroform), and in fractions 12 and 13 (eluted with ethyl acetate and chloroform); steroids were isolated in fractions 6-11 (eluted with petroleum ether and chloroform) and also in fractions 24-26 (eluted with ethyl acetate and chloroform). Further eluting with ethyl acetate and methanol in varying concentrations, the mixture yielded flavonoid in fractions 31-35, glycoside in fractions 28-30, 40-44 and finally steroid again in fractions 45-52. Each fraction was tested for their nature by performing qualitative phyto chemical tests, simultaneously. The fractions 14-23, 27 and 36-39 were mixtures of compounds.

Preparative TLC (for Identification of active component)

In fraction No. 13, terpenoid was identified as ursolic acid (R_f value =1), in fraction No. 24-26, steroid

Table 2 Preparative TLC: Identification of terpenoid, steroid and glycoside by comparison with commercially available standards

SI No	Fraction No	Phytochemical Result (test)	Standard used	Mobile phase used	R _f value of standard	R _f value of test
1	13	Terpenoid	Ursolic acid	Methanol: chloroform (7:43)	1	1
2	24-26	Steroid	β sitosterol	Benzene : petroleum ether: chloroform: ethyl acetate (4:2:3:1)	0.59	0.59
3	28-30	Glycoside	Sitosterol-3-O-glucoside	Benzene : chloroform: ethyl acetate: methanol (18:2:1:4)	0.35	0.35

as β sitosterol (R_f value = 0.59) and in Fraction No.28-30, glycoside as Sitosterol-3-O-glucoside (R_f value = 0.35) (Table 2 and Fig 2).

¹H NMR study of the fractions

The ¹H NMR results of ursolic acid, β sitosterol and sitosterol-3-O-glucoside are shown in Fig -3, Fig - 4 and Fig -5 respectively.

Five main signal peaks are observed in case of ursolic acid. The highest signal peak (doublet) is observed at 1.293 and 1.254 intensities, and chemical shift between 1.5 to 1.1 ppm. In case of β sitosterol, five doublets, one doublet-of-doublets and one triplet is observed, representing that the protons are arranged in three different coupling patterns. Some peaks in this NMR are at 5.823, 4.941, 4.307 etc signal intensities.

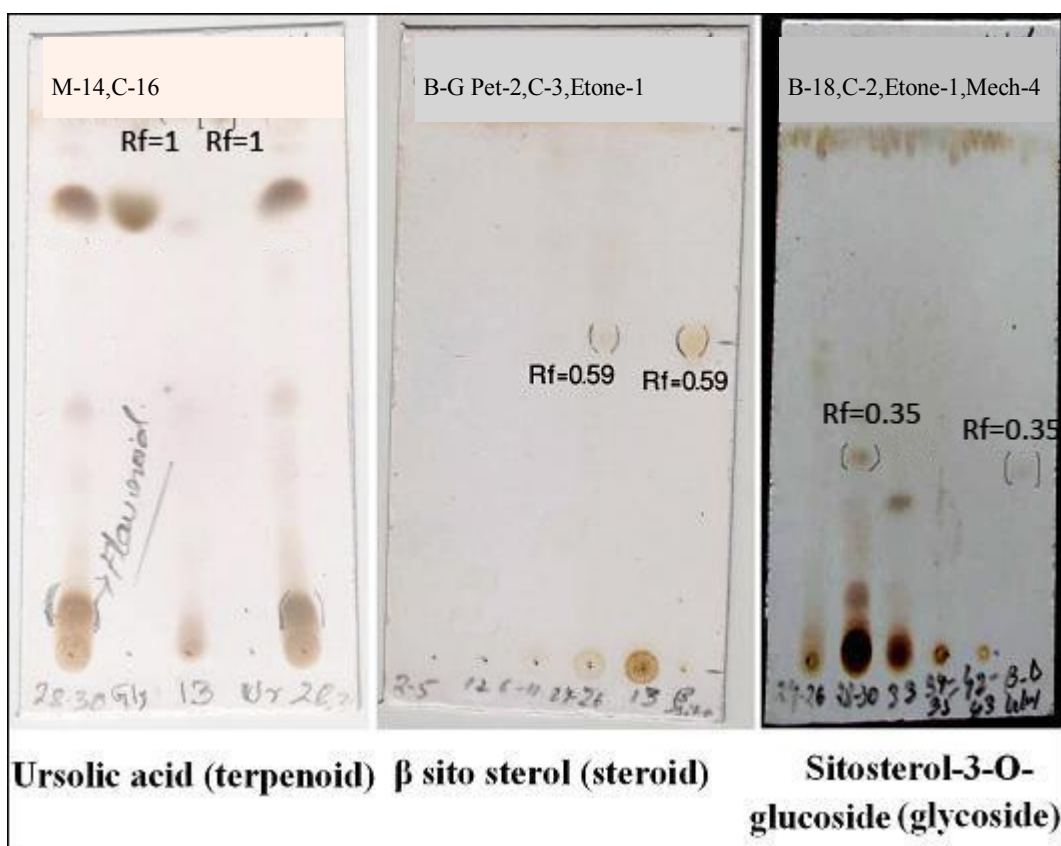


Fig 2 Identification of terpenoid, steroid and glycoside by comparison with standards by TLC (the corresponding R_f values are shown)

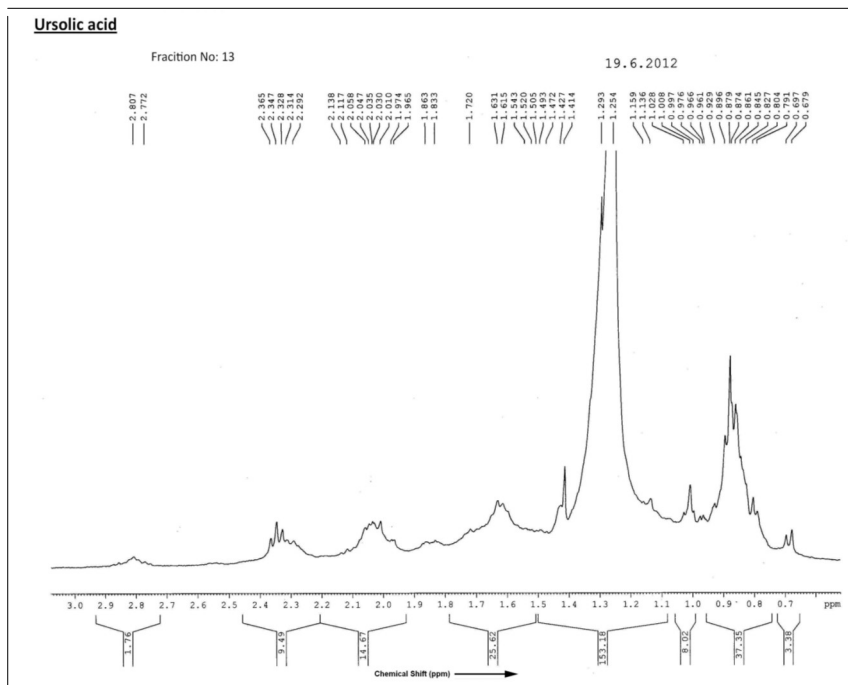


Fig 3 1H NMR study of ursolic acid

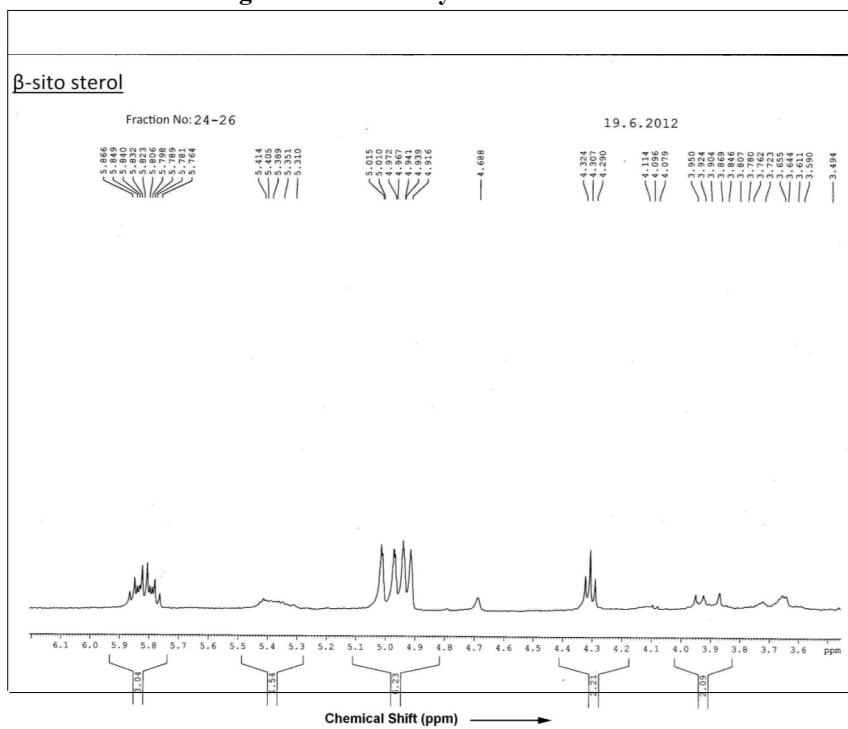


Fig 4 1H NMR study of beta sitosterol

Thirdly, the NMR data of Sitosterol-3-O-glucoside and Sitosterol-3-O-glucoside (Mizushina *et al.*, 2006) are reveals three peaks, among which, the highest signal shown in Fig -6, Fig -7 and Fig-8 respectively. intensity is 1.254 between 71.30 ppm chemical shifts.

Chemical structure of the active components

The chemical structures of ursolic acid (Furtado *et al.*, 2008), beta sitosterol (<http://webprod.hc-sc.gc.ca>)

DISCUSSION

Ursolic acid is a pentacyclic triterpenoid and is a major component of traditional medicinal herbs

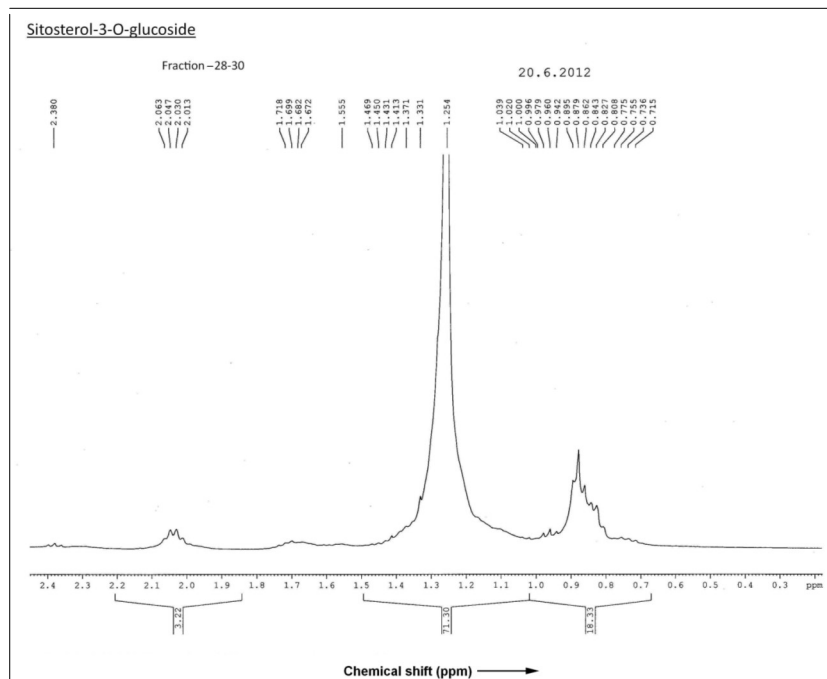


Fig 5 ^1H NMR study of Sitosterol-3-O-glucoside

(Lai *et al.*, 2007). The five signal peaks obtained in our NMR result represents its pentacyclic nature. The highest peak which is a doublet represents the single proton of the OH group which is near the = O group at the right hand side of Fig (vi). The other weak signal peaks are for the protons at the side chains. This triterpenoid has shown anti-ulcer (Ovesná *et al.*, 2004), anti-inflammatory, analgesic (Vasconcelos *et al.*, 2006) anti oxidant (Jun *et al.*, 2010) activities etc. It has potent anti tumor activity also (Furtado *et al.*, 2008). Ursolic acid acts on different stages of tumor

development as tumor initiation and promotion and also plays an important role in tumor cell differentiation apoptosis (Furtado *et al.*, 2008). Pathak *et al.*, (2007) had shown that this terpenoid inhibits STAT 3 activation pathway, leading to the suppression of Human Multiple Myeloma Cells. Subbaramaiah *et al.*, (2000) proved that ursolic acid suppresses the activation of *COX-2* gene expression by inhibiting the PKC signal transduction pathway.

β sitosterol is widely available in a variety of plant and plant parts. The five doublets, one doublet of

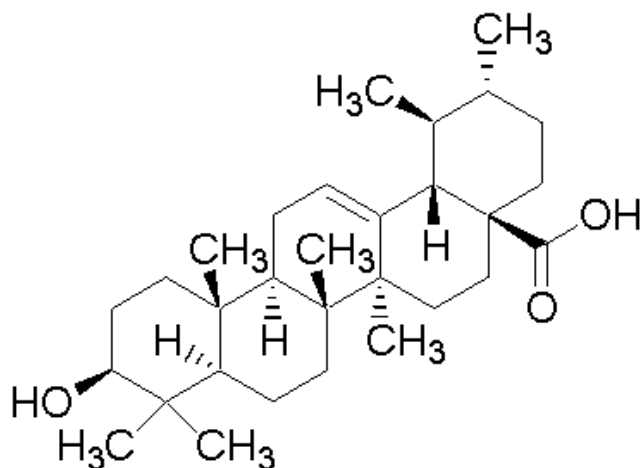


Fig 6 Structure of ursolic acid

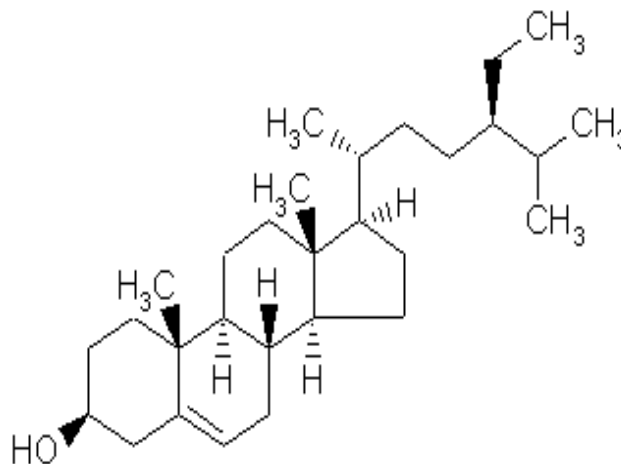


Fig 7 Structure of β sitosterol

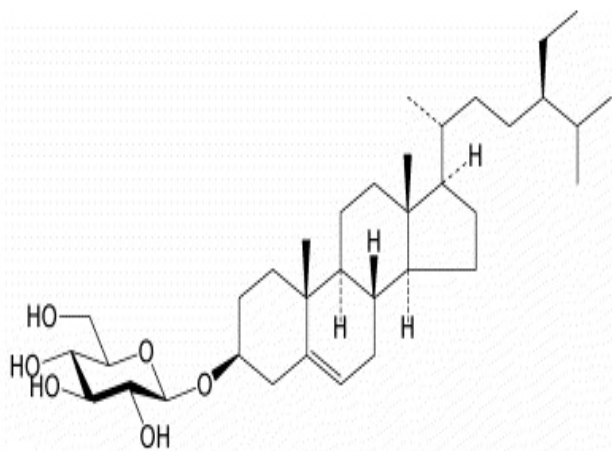


Fig 8 Structure of Sitosterol-3-O-glucoside

doublets and one triplet is observed in our NMR study of in this steroid represent the hexacyclic and pentacyclic groups; the connecting chain and the methyl groups respectively. This sterol is reported in the treatment of benign prostatic hyperplasia (Berges *et al.*, 1995). Carine *et al.*, (2006) proved that beta-sitosterol in combination with polyphenols from cocoa inhibits proliferation of prostatic cancer cell growth.

Sitosterol-3-O-glucoside falls under the class β D glycoside. The three peaks of Sitosterol-3-O-glucoside our NMR study represent the gluco-pyranose sugar, cyclo-hexagon and cyclo-pentagon groups and the free side chains (Fig (viii)). Gohar *et al.*, (2009) had reported that methanolic extracts of seeds of *Ceratonia siliqua* L. has a rich source of natural anti oxidants, which contains β -sitosterol-3-O- β -D-glucoside along with other flavonol glycosides.

In the present work, we are the first to report the rich source of ursolic acid, β sitosterol and sitosterol-3-O-glucoside in leaves and other parts of the plant, *Barleria lupulina*. It is discussed above that ursolic acid and β sito sterol show potent anti cancer activity and sitosterol-3-O-glucoside has anti oxidant activity. Leaf extract of *Barleria lupulina* containing these components, has been found by us to have anti clastogenic and anti tumor activity (in mice and fish)

which had been discussed in our previous work (Sur and Das, 2012). Therefore in conjugation with ursolic acid, these components together act as a potent anti cancer agent in mice and fish models. We also report the anti cancer activity in ethanolic extracts of leaves of *Barleria lupulina*, due to the presence of ursolic acid; for the first time.

Anticlastogenic, radio-protective, anti-tumor and anti-cancer activities from this plant extract has been applied to Govt. of India for patenting, by us (Sur and Das, 2012).

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