Carbonic anhydrase inhibitory potential of *Lagenaria siceraria* and identification of its bioactive compounds-An LC-MS/MS approach

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ABSTRACT

Lagenaria siceraria Stand is an important member of Cucurbitaceae family, widely consumed as a vegetable in daily food habits among Indians. The current study was aimed to identify the active chemical compounds of *L. siceraria* involved in carbonic anhydrase inhibition through bioassay and liquid chromatography-mass spectrometry guided analysis. The extraction of fruits of *L. siceraria* was carried out in methanol. Fractionation of the methanolic extract *L. siceraria* (LSME) was performed in three different solvents (hexane, ethyl acetate and water) successively. The carbonic anhydrase inhibitory activity was evaluated through its esterase inhibition assay. Liquid chromatography and mass spectroscopy (LC-MS/MS) analysis of the bioactive fraction was performed to identify the major bio-actives present in it. The carbonic anhydrase inhibition assay revealed that the LSAF (aqueous fraction) possess highest esterase inhibition activity. The IC_{50} value of LSAF was found equipotent activity with acetazolamide. LC-MS/MS analysis of LSAF revealed the presence of phenolic compounds, confirmed by their MS/MS spectrum. This finding suggests that phenolic compounds of *L. siceraria* may be responsible for carbonic anhydrase inhibition activity.

INTRODUCTION

Lagenaria siceraria Stand popularly known as Bottle gourd is an important food plant of cucurbitacea family. It is very well conversed in Ayurveda and folk medicine for its some potential therapeutic purposes. The phyto-constituents found in the fruit includes cucurbitacin B, phenolic glycosides, flavonoids, flavon-C-glycoside such as isovitexin, isoorientin, saponarin, phenolic acids like caffeic acid, cinnamic acid, flavonoids, sterols like fucosterol,campesterol, cytotoxic polysaccharides (Gangwal et al., 2010, Jaiswal et al., 2014). The plant possesses several significant therapeutic potentials include diuretic, antioxidant, antihyperglycemic, antihyperlipidemic, cardioprotective activity, immunomodulatory, anthelmintic activity, (Ghule et al., 2009). Several phenolic compounds (phenolic acids, flavonoids) are considered beneficial for their activity. Carbonic anhydrase is a metalloenzyme, is involved in several pathophysiological processes in human. The present work was undertaken to establish the carbonic anhydrase inhibitory property of the bioactive fraction of LS and identifying its major phytoconstituents by LC-MS/MS study. This strategy was found to be very useful for rapid identification of bioactive components in plant extract prior to isolation of the pure compounds.

MATERIALS AND METHODS

Carbonic anhydrase II from bovine erythrocytes (3848 W/A units/mg solid) (EC-232-576-6) (bCA II) and p-nitrophenyl acetate (p-NPA) was purchased from Sigma Aldrich, St. Louis, MO, USA. Acetazolamide IP (Batch No. AZM-V-P/131107) was procured as a gift sample from Mangalam Drugs and Organics Ltd., Mumbai, India.
Other chemicals and solvents were purchased from Merck Pharmaceuticals, Mumbai.

In-vitro carbonic anhydrase activity:
In-vitro carbonic anhydrase inhibition assay was performed through esterase inhibition model (Verpoorte et al., 1967). The absorbance of the samples in each well was determined at 400 nm using a UV-visible spectroscopy (SpectraMax Plus, United States). The change of absorbance was observed due to the liberation of p-nitrophenol as the hydrolysis product of p-NPA. The assay procedure was carried out in triplicate. Acetazolamide was used as a positive control. Relative carbonic anhydrase activity (%) = (catalytic rate of esterase reaction with inhibitor)/ (catalytic rate of esterase reaction without inhibitor) × 100. IC50 values of inhibitors were determined by plotting the percentage of enzyme activity against the inhibitor concentration (Bijari et al., 2015).

HPLC Conditions:
Chromatographic separation was performed on LC800 (GL Sciences). The auto-sampler and column heater temperature was maintained at 25ºC and the injection volume was set at 15 µL for all analyses. The chromatographic separation was achieved on Agilent Zorbax Eclipse C18 column (50× 2.1mm, 1.7µm). The mobile phases consisted of acetonitrile (A)and water (B) both containing 0.1% formic acid. The gradient profile was set at 10% B from 0 to 1 min, 30% B at 8 min, 40% B at 12 min, 80% B at 16min, 95% B from 20-27 min, and finally 10% B at 28-35min. The flow rate for all separations was set at 0.7 mL/min.

MS/MS Conditions:
The sample was analyzed in hybrid TripleTOF® 5600 fitted with a DuoSpray™ ion source (AB Sciex, Concord, Canada). Every sample was injected twice in positive and negative polarity. The instrument was set to perform one TOF MS survey scan (150 ms) and 20 MS/MS scans (50 ms each) with a total duty cycle time of 1.2 s. The mass range of both scan types was 50-1000 m/z. Acquisition of MS/MS spectra was controlled by IDA function of the Analyst® TF software (AB Sciex, Concord, Canada).

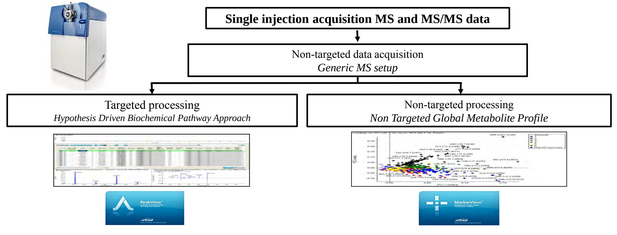


Figure 1. Workflows for targeted and non targeted analysis

RESULTS

Effect of *L. siceraria* on carbonic anhydrase activity:
The LS metanolic extract (LSME) and three other fractions (aqueous, ethyl acetate, hexane) were assayed for carbonic anhydrase inhibition activity. The IC50 values of aqueous fraction (LSAF) and acetazolamide (positive control) was estimated to be 403.34 ± 5.04 µg/mL, 220.32 ± 3.56 µg/mL respectively. (Fig. 1). It has been found that, LSAF inhibited carbonic anhydrase activity up to 89% at the concentration of 900 µg/ml in dose dependent manner.(Fig. 2). The result also suggested that LSAF inhibited carbonic anhydrase, reversibly. The enzyme kinetic analysis revealed mixed inhibition mode (i.e., both competitive and non-competitive) of LSAF, further supported by Lineweaver-Burk plot analysis of the kinetic data.

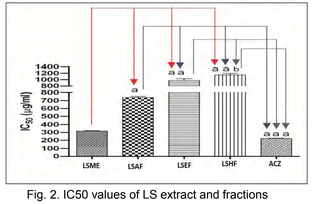


Fig. 2. IC50 values of LS extract and fractions

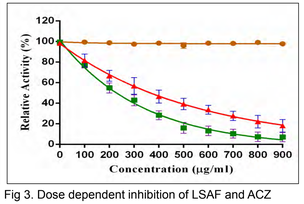
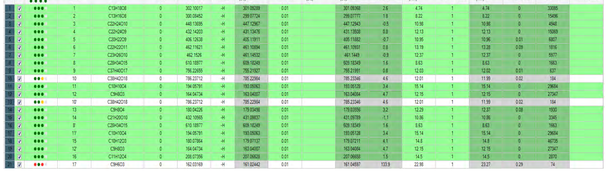


Fig 3. Dose dependent inhibition of LSAF and ACZ

Identification of chemical compounds



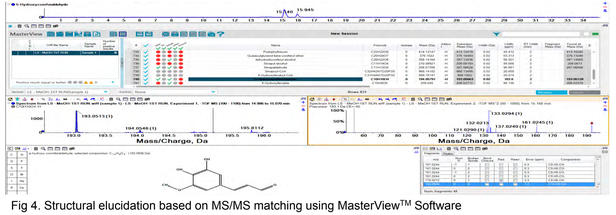


Fig 4. Structural elucidation based on MS/MS matching using MasterView™ Software

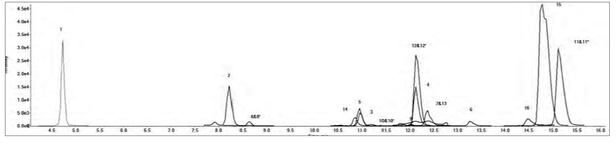
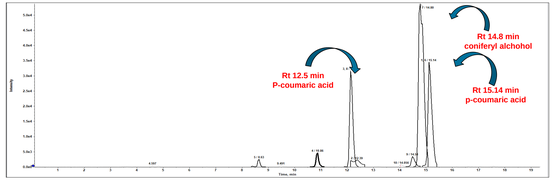


Fig 5. List of major phytoconstituents in LSME

Peak	Rt	Formula	Mass (Expt.)	Mass (Theor.)	Error (ppm)	Isotopic diff.	Proposed compound
1.	4.74	C13H18O8	301.0937	301.0929	2.6	3.6	4-O-glucosyl-3,4-dihydroxybenzyl alcohol
2	8.22	C13H16O8	299.0778	299.0772	1.8	7.6	4-O-glucosyl-4-hydroxybenzoic acid
3	10.98	C22H24O10	447.1294	477.1297	-0.5	0.9	4-O-(6'-O-glucosylcaffeoyl)-4-hydroxybenzyl alcohol
4.	12.13	C22H24O9	431.1351	431.1348	0.8	1.3	4-O-(6'-O-glucosyl-p-coumaroyl) -4-hydroxybenzyl alcohol
5.	10.95	C20H22O9	405.1188	405.1191	-0.7	2.8	4-O-(6'-O-glucosyl-4"-hydroxybenzoyl)-4-hydroxybenzyl alcohol
6.	13.19	C22H22O11	461.1089	461.1089	0	1.1	4-O-(6'-O-glucosylcaffeoyl)-4-hydroxybenzoic acid
7.	12.37	C23H26O10	461.1449	461.1453	-0.9	0.4	4-O-(6'-O-glucosylferuloyl)-4-hydroxybenzyl alcohol
8.	8.63	C28H34O15	609.1835	609.1825	1.6	1.8	4-O-(6'-O-glucosylcaffeoylglucosyl)-4-hydroxybenzyl alcohol
9.	12.03	C37H40O17	755.2202	755.2193	1.2	2	4-O-(6'-O-glucosylcaffeoylglucosylp-coumaroyl)-4-hydroxybenzyl alcohol
10.	12.01	C38H42O18	785.2335	785.2298	4.6	4.9	4-O-(6'-O-glucosylferuloylglucosylcaffeoyl)-4-hydroxybenzyl alcohol
11.	15.14	C10H10O4	193.0513	193.0506	3.4	0.50	Ferulic acid
12.	12.15	C9H8O3	163.0408	163.0401	4.7	1.10	p-Coumaric acid
13.	12.29	$C_{9}H_{8}O_{4}$	179.0355	179.035	3	0.50	Caffeic acid
14.	10.86	C21H20O10	431.0979	431.0984	-1.1	4.1	genistin
15.	14.8	C10H12O3	179.0721	179.0714	4.1	0.90	Coniferyl alcohol
16.	14.5	C11H12O4	207.0666	207.0663	1.5	5.90	Sinapaldehyde

Table 1. List of compounds identified



Chemical compounds present in most bioactive sub-fractions of LSAF

Retention time	Compounds
12.15 min	p-coumaric acid
14.8 min	Coniferyl alchohol
15.14 min	Ferulic acid

CONCLUSIONS

The carbonic anhydrase inhibitory activity assay of *L. siceraria* methanolic extract and fractions were performed. The aqueous fractions was found maximum inhibition with compared to other fractions due to highest phenolic content, which was further confirmed by LC-MS/MS analysis. The enzyme kinetics study revealed the inhibition as reversible and mixed in nature. From these findings, it can be concluded that, the phenolic compounds present in *L. sicearia* may be served as carbonic anhydrase inhibitors with possible therapeutic benefits. The MasterView™ software (AB Sciex, Concord, Canada) offers an easy to use and intuitive workflow to identify bioactive compounds with high precision.

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TRADEMARKS/LICENSING

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