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Research Article

Carbonic Anhydrase I and II Inhibition with Natural Products: *Leucas cephalotes*

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ABSTRACT: Carbonic anhydrases (EC 4.2.1.1) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes that are encoded by five evolutionarily unrelated gene families are involved in numerous physiological and pathological processes. Novel interesting chemo types, in addition to the sulphonamide and sulfamate were discovered, many of which are based on natural products, such as phenols/polyphenols, phenolic acids, and coumarins. Methanolic extract of *Leucas cephalotes* belongs to family Labiatae (or Lamiaceae) tested for human carbonic anhydrase (hCA) I and II inhibition study. The significant IC 50 values are calculated for the methanolic extract of *Leucas cephalotes* for hCA I found to be 0.23 mM/ml which is showing relatively less potent then hCA inhibition against hCA II having IC 50 values of 0.19 mM/ml. *Leucas cephalotes* is a weak inhibitors they may constitute leads for developing tighter binding compounds.

KEYWORDS*:* Carbonic anhydrase; Enzyme inhibitor; Natural product; *Leucas cephalotes*.

INTRODUCTION

Carbonic anhydrases (CAs; also known as carbonate dehydratases EC 4.2.1.1) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes that are encoded by five evolutionarily unrelated gene families. These are the α-CAs (present in vertebrates, bacteria, algae and cytoplasm of green plants); the β-CAs (predominantly in bacteria, algae and chloroplasts of monocotyledons and dicotyledons); the γ-CAs (mainly in archaea and some bacteria); and the δ-CAs and ζ-CAs (present in some marine diatoms).[1-5] In mammals, 16 α-CA isozymes or CA-related proteins with different catalytic activity, subcellular localization and tissue distribution are there.[6-13]

CAs catalyse a simple physiological reaction the conversion of CO_2 to the bicarbonate ion and protons. The active site of most CAs contains a zinc ion (Zn^{2+}), which is essential for catalysis. The CA reaction is involved in many physiological and pathological processes, including respiration and transport of CO_2 and bicarbonate between metabolizing tissues and lungs; pH and CO_2 homeostasis; electrolyte secretion in various tissues and organs; biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis); bone resorption; calcification; and tumorigenicity.[6-9]

Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat a range of disorders including oedema, glaucoma, obesity, cancer, epilepsy and osteoporosis. Two main classes of CA inhibitors (CAIs) are known: the metal-complexing anions and the unsubstituted sulphonamides and their bioisosteres -for example, sulphamates and sulphamide compounds.[1-26] These inhibitors bind to the Zn^{2+} ion of the enzyme either by substituting the non-protein zinc ligand to generate a tetrahedral adduct or by addition to the metal coordination sphere to generate a trigonal bipyramidal species.[1-5]

Recently, novel interesting chemotypes, in addition to the sulfonamide and sulfamate were discovered, many of which are based on natural products, such as phenols/polyphenols, phenolic acids, and coumarins. Their detailed mechanism of inhibition has been explained by means of kinetic and X-ray crystallographic studies and can be used for the rational drug design of other agents.[7-11]

Structure A-G

The X-ray crystal structure of the physiologically dominant isoform, CA II, in adduct with phenol **F** has been reported

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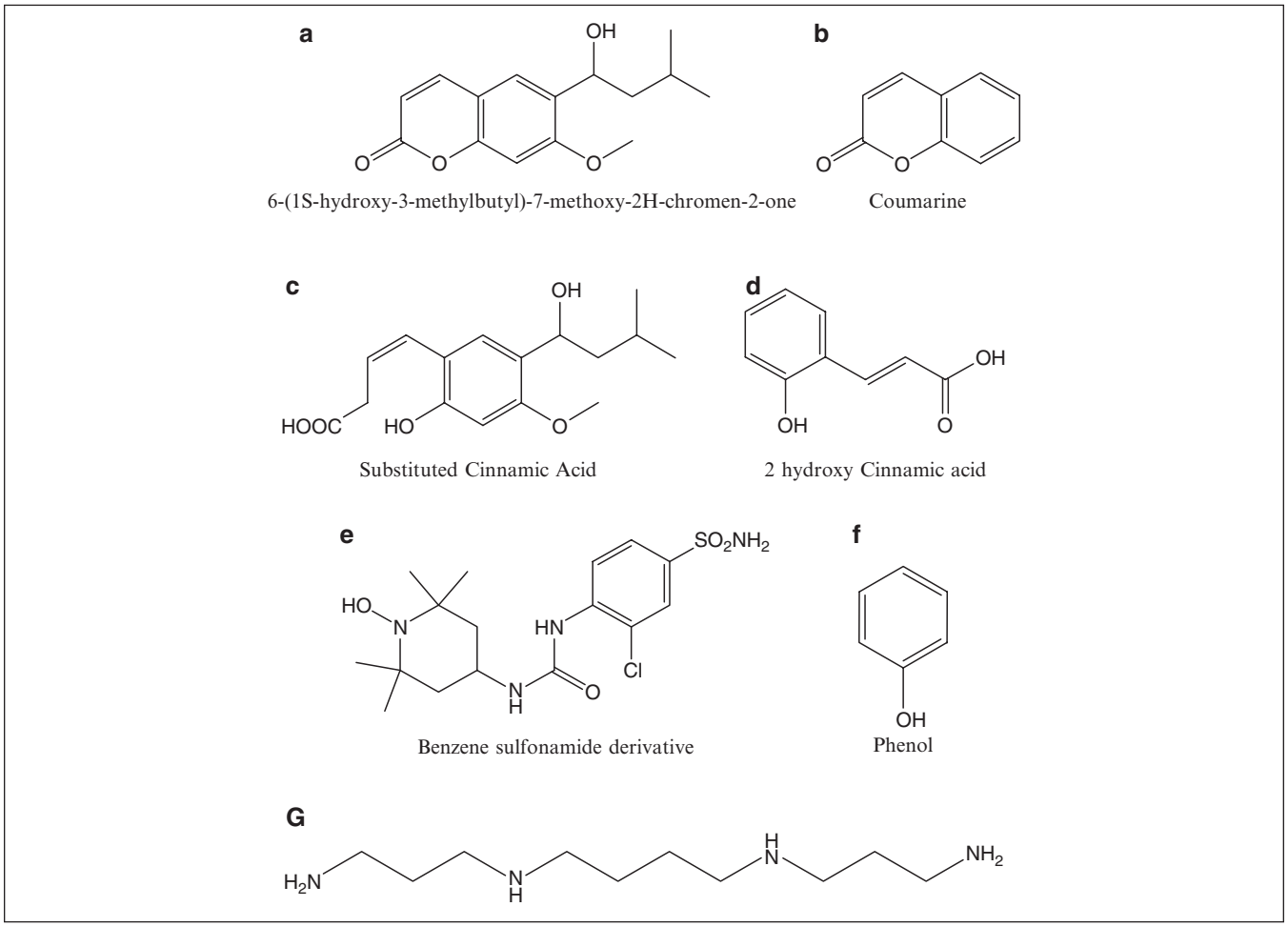

by Hristianson's group.[10] As outlined in phenol **F** was found anchored by a hydrogen bond by means of its OH moiety, to the fourth zinc ligand which is a water molecule, or a hydroxide ion shown in Figure 1 (a).

Figure 2 represent distances (in Å). Hydrogen bonds are represented as *dashed lines*. Two classes (C5 and C7) of the spermine scaffold with a water molecule (Wat113) and Gln92 had shown in bold. The non-protein zinc ligand is represented as a hydroxide ion, which should be the preponderant species at the pH at which the experiments were done.[26]

It should also be noted that coumarins **A** and **B** and hydrolyzed coumarins **C** and **D** were potent inhibitors against some investigated human CA isoforms, which makes this entire class of derivatives of paramount interest for designing novel applications for the CAIs. The binding of the hydrolyzed coumarins **C** and **D** to hCA II is shown in Figure 1 (b), where the structures of a benzene sulphonamide CAI **E** and of simple phenol **F** are also presented, stressing the novelty of the binding mode of this chemotype to the enzyme, in comparison to the classical inhibitors (sulphonamides, which interact with the zinc ion) or phenols **F** (which interact with the zinc-coordinated water molecule).[13,16]

MATERIALS AND METHODS

Apparatus and Chemicals

Soxhlet apparatus, heating mantle, Vacuum evaporator, SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow, Methanol (methyl alcohol, Central Drug House: 015127, HPLC grade), petroleum ether, Hger's resgents (Picric acid), Mayer's reagent (potassiomercuric iodide solution), Wagner's reagent (solution of iodine in potassium iodide), Dragendorff's reagent (potassium bismuth iodide), ferric chloride solution, Iodine solution, Fehling's solution, Ninhydrin solution, 1% copper sulphate solution, 10% sodium hydroxide solution, phenol red (0.2 mM), 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$, Saturated CO_2 solutions in water at 25 °C, DMSO-water 1:1, v/v.

Plant Materials

Leucas Cephalotes (Roth) Spreng. Syn. Phlomis cephalotes (Labiatae or Lamiaceae) rainy season weed mainly found

in North India. It is commonly known as 'Kubo or Kubi' in traditional medicine of Gujarat. The genus Leucas includes about 100 Asiatic and African species. The whole plants of *Leucas cephalotes* were collected from GITAM University campus, Visakhapatnam, Andhra Pradesh, India. The herb collected in the morning and photo (Figure 3) taken by Dr. Kalyan Kumar Sethi, Assistant Professor, GITAM University, Visakhapatnam, India. The plant was authenticated by Dr. Posa Mahesh Kumar, Assistant Professor, GITAM University, India.

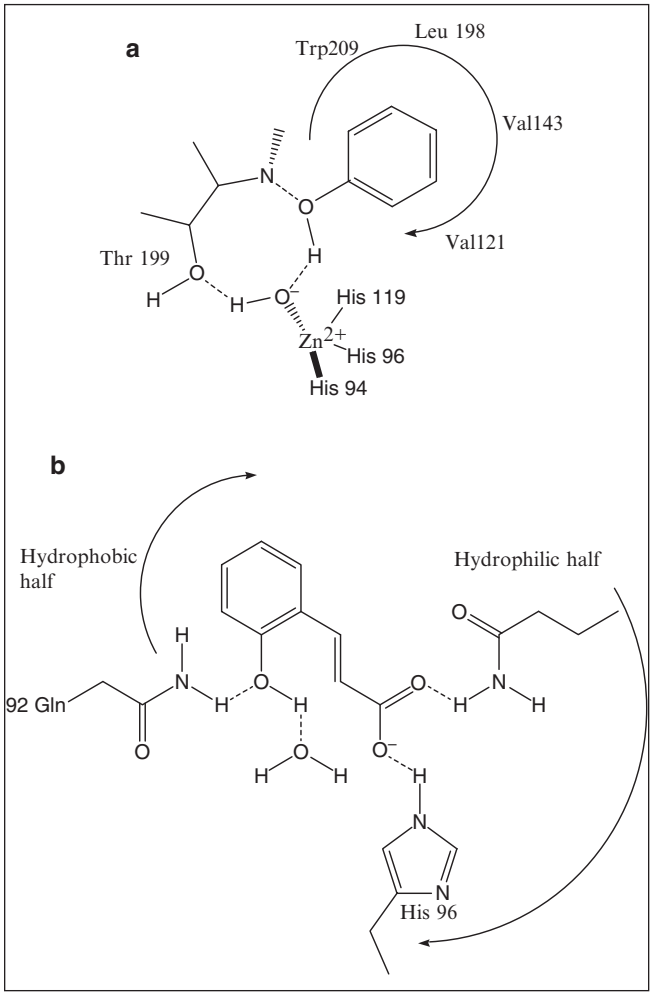

Figure 1: (**a**) Phenols **F** anchor to the Zn (II) coordinated water molecule/hydroxide ion;[26] (**b**) Coumarins (hydrolyzed in situ to 2-hydroxycinnamic acids) occlude the entrance of the active site cavity, interacting both with hydrophilic and hydrophobic amino acid residues. The inhibitor does not interact at all with the catalytically crucial Zn (II) ion which is coordinated by three His residues and a water molecule.[25,24]

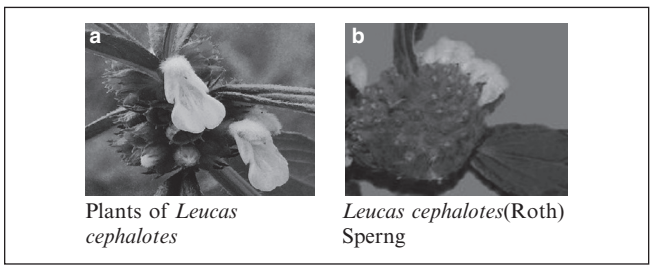

Figure 3: *Leucas cephalotes:* plant and seeds.

Extraction of Plant Materials

Powder of *Leucas cephalotes* was was first defatted with petrolium ether. Then it was extracted by methanol with the help of soxhlet apparatus from whole plant (Figure 3).[25] The methanolic extract which is further evaporated to dryness to obtain alcoholic extract.

Phytochemical Screening

All kinds of qualitative identification test have been performed to identify the type of compounds present in it which described in table 1. The extracts obtained from methanolic solvent extraction were then subjected to various qualitative chemical tests to determine the presence of various phytoconstituents like alkaloids, glycosides,

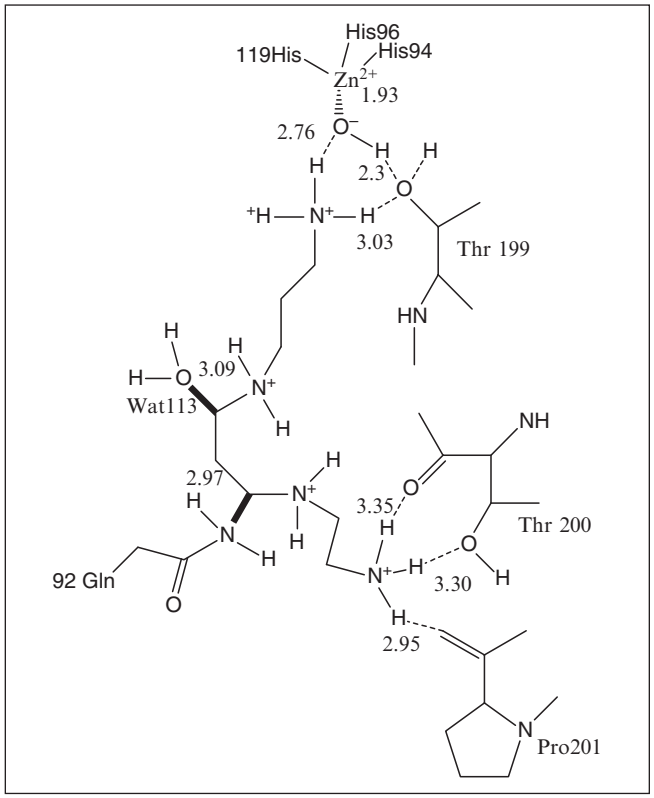

Figure 2: Schematic representation for interactions in which spermine **G** (as tetracation) participates when bound to the hCA II active site.

Table 1: Qualitative identification test for methanolic extract of *Leucas cephalotes*

Test	Identification
Alkaloids	–
Glycosides	–
Carbohydrates	+
Phytosterols	+
Saponins	–
Phenolic compounds	+
Proteins and amino acids	–
Flavanoids	+

carbohydrates, phenolics, phytosterols, proteins an amino acids, flavonoids and saponins.[32]

Qualitative identification test of *Leucas cephalotes* shown +ve for the evidence of having flavanoids, phytosterols, **phenolics** and carbohydrates components. Which indicate the presence of flavanoids open evidence for structural similarity of functional group present in coumarine. The presence of phenolic compounds in this satisfies the mechanism whown in fig-1. Based on this evidence *Leucas cephalotes* create a novel interest for the study for hCA inhibitors. Considering the above fact the extract of *Leucas cephalotes* tested for hCA I and II inhibition study.

Enzyme inhibition study of *Leucas cephalotes* on hCA I and hCA II

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[27] Phenol Red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration), following the CA-catalyzed CO_2 hydration reaction for a period of 5-10s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO-water 1:1, v/v) and dilutions up to 1 mM done with the assay buffer mentioned above. At least 4 different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier, and represent the mean from at least three different determinations. All CA isozymes used here were recombinant proteins obtained as reported earlier by our group.[29-31] As seen in tables, several millemolar CAIs against the cytosolic isoforms hCA I and hCA II, have been determined.

RESULTS

The extracted *Leucas cephalotes* is tested for hCA I inhibition study. Various mille equivalent of extract of *Leucas cephalotes* are prepared and compared with blank inhibitor concentration which shown in Table 2. The IC 50 values of extracted *Leucas cephalotes* against hCA I is found to be 0.23 mM/ml. The graph explain the % inhibition of hCA I against the concentration is plotted between percentage activity and concentration which shown in Figure 4.

The extracted *Leucas cephalotes* is tested for hCA II inhibition study. Various mille equivalent of piperine are prepared and compared with blank inhibitor concentration which shown in Table 3. The IC 50 values of *Leucas cephalotes* against hCA II is found to be 0.19 mM/ml. The graph

Table 2: Enzyme inhibition of *Leucas cephalotes* on hCA I

Comp.	Inhibitor conc.	Slope	%Free Enzyme	% Inhibition	IC 50
hCAI 10-6M	0	3.31	100%	0%	0.23 mM/ml
Leucas Cephalotes	2.0E-03	2.87	87%	13%	
Leucas Cephalotes	2.0E-02	2.12	64%	36%	
Leucas Cephalotes	2.0E-01	1.26	38%	62%	
Leucas Cephalotes	2.0E+00	0.39	12%	88%	

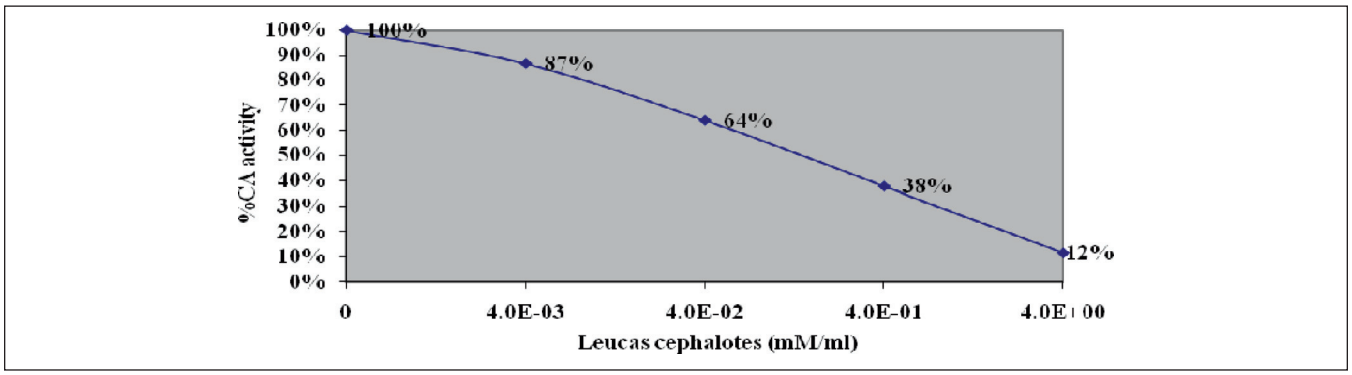

Figure 4: Graph of % inhibition of CA I and concentration of *Leucas cephalotes* as inhibitor

Table 3: Enzyme inhibition of *Leucas cephalotes* on hCA II

Compound	Inhibitor conc.	Slope	% Free Enzyme	% Inhibition	IC 50
hCAII	0	43.78	100%	0%	0.19 mM/ml
10-6M					
Leucas Cephalotes	4.0E-03	36.47	83%	17%	
Leucas Cephalotes	4.0E-02	26.59	61%	39%	
Leucas Cephalotes	4.0E-01	15.82	36%	64%	
Leucas Cephalotes	4.0E + 00	5.411	12%	88%	

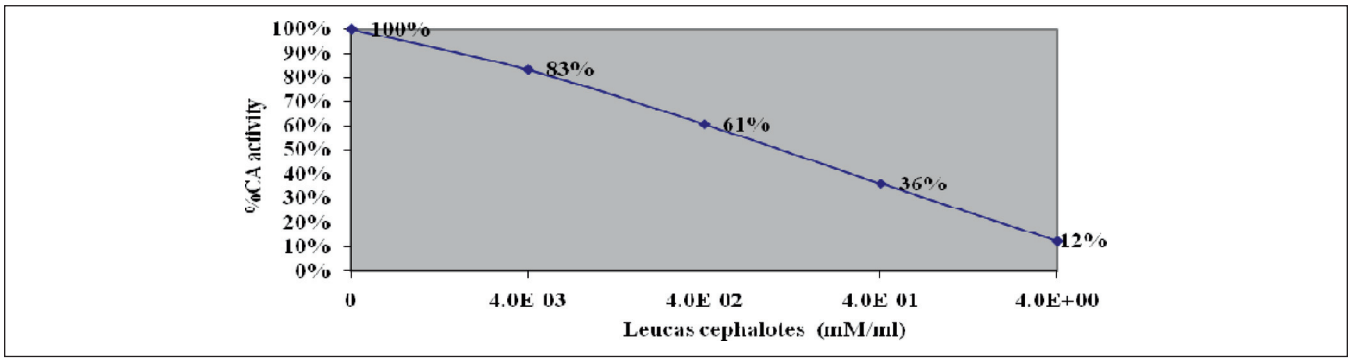

Figure 5: Graph of % inhibition of CA II and concentration of *Leucas cephalotes* as inhibitor

explain the % inhibition of hCA II against the concentration is plotted between percentage activity and concentration which shown in Figure 5.

DISCUSSION

Acetazolamide which taken as the reference compound have the IC 50 values against hCA I and hCA II were 0.35×10^3 mM/ml and 0.24×10^4 mM/ml respectively. The IC 50 of *Leucas cephalotes* against hCA I is found to be 0.23 mM/ml which is showing relatively less potent then hCA inhibition against hCA II having IC 50 values of 0.19 mM/ml.

CA I is a cytisolic form mainly found in Erythrocytes, GI tract. The slow cytosolic isozyme CA I mediates haemorrhagic retinal and cerebral vascular permeability through activation of prekallikrein and generation of the highly active serine protease factor XIIa. These phenomena contribute to the pathogenesis of proliferative diabetic retinopathy and diabetic macular oedema, which represent leading causes of vision loss, and for which there are no pharmacological treatments currently available. Therefore, as suggested by the authors, CA I inhibition might be a therapeutic target for the treatment of these conditions. In fact, potent CA I inhibitors are currently available.

CA II found in erythrocytes, eye, GI tract, bone osteoclasts, kidney, lung, testis, brain. CA II inhibitors penetrated the cornea and significantly lowered Intra Ocular Pressure (IOP) in both normotensive and glaucomatous rabbits. Inhibition of cytosolic (CA II) enzymes seems to be involved in the diuretic effects of the *Leucas cephalotes*. CAs was identified in the anterior uvea of the eye and was shown to be responsible for the bicarbonate secretion. CAIs represent the most physiological treatment of glaucoma, as by inhibiting the ciliary-process enzyme — the susceptible isozyme CA II — the rate of bicarbonate and aqueous humour secretion is reduced, resulting in a 25-30% decrease in IOP.

The phenolic compounds present in *Leucas cephalotes* satisfy the mechanism (figure 1). Phenols and their derivatives compose a class of scarcely investigated CAIs with great promise. Unfortunately, there is little information on how this class of CAIs bind to the enzyme active site to allow for structure-based drug design campaigns. Considering the high number of phenolic natural products present in nature, this class of derivatives may lead to novel chemotypes with CA inhibitory properties and warrant further investigations.

CONCLUSION

Leucas cephalotes is weak inhibitors they may constitute leads for developing tighter binding compounds and may create a novel interest, in addition to the sulphonamide and sulfamate and other natural products such as phenols/polyphenols, phenolic acids, and coumarins were discovered. The new applications of CAIs range from antiglaucoma agents with topical activity, to anticonvulsants, antipain, antiobesity, and antitumor agents/diagnostic tools for cancer. This idea is not widely accepted, there is potential to develop anti-infectives (antimalarials, antifungal, and antibacterial

agents) belonging to the CAIs, targeting enzymes from various pathogens. It is thus, foreseeable that novel therapeutic applications will emerge for this Natural Product based enzyme inhibitors in the near future.

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