Pharmacokinetic properties of inhibitors to Histidine Decarboxylase isolated from fennel

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ABSTRACT
Histamine release is involved in developing wakefulness and activation of cortex. The purpose of the study was to find an alternative to these antihistamines by inhibiting the enzyme histidine decarboxylase which is responsible for the production of histamine. Histamine brings about the allergic response due to mast cell degranulation. It is also necessary that the enzyme is not completely inhibited as it plays a role as a neurotransmitter and also regulates gastric acid secretion. In the present study, the methanolic extract of commercially available fennel seeds were examined for its inhibitory activity on the purified extract of bacterial histidine decarboxylase. The methanolic extract of fennel was analyzed to check the presence of various phytochemical constituents. The spice extract was quantified to estimate the presence of flavonoids. The extract was subjected to purification by thin layer chromatography and HPLC in order to isolate and identify the flavonoids present in spice extract. The HPLC results with reference to the standard indicated the presence of ellagic acid and quercetin. The spice extracts were subjected to inhibitory studies at increasing concentrations. The fennel extract at concentration of 0.625 moles was found to be inhibiting histidine decarboxylase which was determined using the Dixon plot. The identified flavonoids were then subjected to software’s like Molinspiration and Swiss ADME in order to study the molecular properties, drug likeliness and pharmacokinetics.

INTRODUCTION
Traditional spices have always been of great economic importance in a country like India. They possess a wide variety of medicinal properties due to presence of phytochemicals which have the ability to produce a biological effect. Fennel is a perennial herb belonging to the Apiaceae family and largely grown in the temperate regions of the northern hemisphere. It is a highly aromatic herb used for both culinary and medicinal purposes. The dry seed is traditionally used as an anti-inflammatory, analgesic, carminative, diuretic and antispasmodic agent (Agarwal et al., 2007).

Hypersensitivity is the inappropriate, inflammatory response that can have deleterious effects such as tissue injury and even death thus, damaging the host. Histamine plays a major role in type 1 hypersensitivity i.e., IgE mediated hypersensitivity. The allergen – IgE complex formed binds to the high affinity FcεRI receptors to mediate a response that brings about mast cell degranulation releasing mediators, of which histamine is a major component (Kindt et al., 2007).
Histamine is a naturally occurring biogenic amine produced by the decarboxylation of histidine mediated by the enzyme histidine decarboxylase. The present-day antihistamines are seen to produce deleterious side effects such as sedation, drowsiness, muscle weakness etc. An alternate to this can be discovered by the usage of flavonoids present in the traditional spices as inhibitors of histidine decarboxylase.

Recently, the inhibitory effect of traditional spices on amine forming bacteria and decarboxylase activity was reported [Wendakoon and Sakaguchi, 1995]. The presence of spice components in culture medium of Enterobacter aerogenes caused a delay in the production of biogenic amines and higher concentrations were found to inhibit cell growth.

In the present study, the inhibitory effect of fennel extracts was examined on histidine decarboxylase enzyme purified from Enterobacter spp.
0.2-1.0 mL aliquots of the standard quercetin solution was pipetted out. The volume of each tube was made up to 2 mL with methanol. 2.0 mL of methanol was taken as blank. To this reaction mixture, 0.1 mL of 10% AlCl₃ was added followed by 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The tubes were then incubated for 30 minutes at room temperature and the absorbance was read at 415 nm at the end of incubation period. The flavonoid content was measured as mg quercetin equivalent/g of extract (Woisky and Salatino, 1998).

**Purification of flavonoids**

Partial purification of flavonoids by TLC - TLC was performed to partially purify the plant extracts for flavonoids. The solvent system used was n-butane: ethyl acetate: distilled water in the ratio of 5:10:15. The sample was spotted on the TLC plates and was allowed to run with the above solvent system. After completion of the run, the plates were air dried and sprayed with a mixture of 3% boric acid and 10% oxalic acid. The plates were then visualized under the UV transilluminator for green fluorescence spots for the indication of flavonoids (El-Olemy et al., 1994).

**Purification of flavonoids by HPLC**

The HPLC system (Agilent Technologies Company) was equipped with dual lamp binary system, UV detector, C18 column (i.e., 4.6 mm × 150 mm, 5 μm) and data was integrated by Agilent Chem Station software. Standards and sample extracts were analyzed using the following gradient program (A.100% acetonitrile B HPLC Grade Water 0 min, 5%A: 10 min, 15%A: 20 min, 25%A: 30 min, 35%A: 40 min, 45%A: 50 min 55%A). Flow rate was 0.8 mL/min and injection volume were 20 μL. Detection was done at 280 nm. The samples were detected at 280 nm by analyzing the peaks obtained and the retention time of individual peaks was used to identify polyphenols by comparing with standard polyphenols such as caffeic acid, kaempferol, quercetin, gallic acid, ferulic acid and ellagic acid (Dua et al., 2013).

**Inhibition of histidine decarboxylase**

The grown cultures of Enterobacter spp. were subjected to sonication and the resulted enzyme was initially partially purified by ammonium sulphate precipitation. Further purification was carried out by cation exchange chromatography, anion exchange chromatography and Molecular exclusion chromatography. The purified enzyme was subjected to inhibition studies. The enzyme was allowed to interact with the flavonoids present in the spice extracts.

It was then allowed to catalyze the decarboxylation of histamine and its activity was determined. The results were then inferred in order to identify the type of inhibition by varying the concentrations of the enzyme and the substrate keeping the concentration of the inhibitor constant.

**Determination of Insilico pharmacokinetics and bioactivity**

The physiochemical and pharmacokinetic properties of the inhibitors of histidine decarboxylase was evaluated using Molinspiration Cheminformatics server (http://www.molinspiration.com). It
Figure 7: The molecular properties of Ellagic acid.

Figure 8: The molecular properties of Quercetin.

Figure 9: The bioactivity scores of Quercetin.

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RESULTS AND DISCUSSION

The inhibition of the enzyme histidine decarboxylase was studied using flavonoids isolated from methanolic extract of fennel. The flavonoids were purified and were identified prior to inhibition assays. The inhibition assays have proved the ability of the flavonoids to effectively inhibit histidine decarboxylase, thus reducing the formation of histamine.

Phytochemical Screening

Phytochemical screening is a method to detect the presence of various phytoconstituents. Phytochemicals are secondary metabolites produced by a majority of plants which are said to be biologically active. These components help them thrive against harsh conditions and competitors. The presence of proteins, phenolics like flavonoids and terpenoids, coumarins and glycosides were detected in the methanolic extracts of fennel whereas lesser quantities of these were observed in the methanolic extracts of green cardamom. The phytochemical analysis of fennel indicates the presence of proteins, phenolics and glycosides. The phenolics include tannins, terpenoids and flavonoids.

Purification of flavonoids

Swiss ADME is provided by the Swiss Institute of Bioinformatics in order to compute the physicochemical descriptors as well as predict ADME parameters, pharmacokinetic properties, drug like nature and medicinal chemistry friendliness of small molecules to support drug discovery (Daina et al., 2017).

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Another study indicated the presence of flavonoids, phenol, glycosides and terpenoids in the methanolic extract of fennel seeds (Bano et al., 2016).

Purification of flavonoids
Table 1: Physicochemical properties of flavonoids

| Compound Name | MW (g/mol) | Physicochemical Properties | TPSA (Å) | LogP | LogS |
|---------------|------------|---------------------------|----------|------|------|
| Ellagic acid  | 302.19     | H bond acceptors: 8       | 141.34   | 0.79 | -2.94|
| Quercetin     | 302.24     | H bond donors: 4          | 131.36   | 1.63 | -3.16|

Table 2: Pharmacokinetic properties of flavonoids

| Compound Name | GI Absorption | BBP permeability | Pgp substrate | Pharmacokinetics | CYP2C19 Inhibitor | CYP2C9 Inhibitor | CYP2D6 Inhibitor | CYP3A4 Inhibitor | LogKP (cm/s) |
|---------------|---------------|------------------|---------------|------------------|-------------------|------------------|------------------|------------------|--------------|
| Ellagic acid  | High          | No               | No            | Yes              | No                | No               | No               | Yes              | -7.36        |
| Quercetin     | High          | No               | No            | Yes              | No                | No               | No               | Yes              | -7.05        |

Table 3: Drug likeliness of flavonoids

| Compound Name | Lipinski’s model | Ghose’s model | Egan’s model | Muegge’s model | Bioavailability score |
|---------------|------------------|---------------|--------------|----------------|----------------------|
| Ellagic acid  | Yes              | Yes           | No           | No             | 0.55                 |
| Quercetin     | Yes              | Yes           | Yes          | Yes            | 0.55                 |

Partial Purification of flavonoids by TLC – flavonoids are structurally derived from the parent substance flavones. They contain conjugated aromatic systems and thus, show intense absorption bands in UV (Harborne, 1984). TLC was used to determine the presence of flavonoids in spice samples and it was detected under UV light on spraying boric acid: oxalic acid mixture as they exhibit fluorescence (Stalikas, 2007) due to their structure. Fennel extract resulted in one spot of green blue fluorescence, whereas green cardamom showed a faded blue fluorescence [Figure 1]. Due to the decreased presence of flavonoids as indicated by phytochemical screening and quantification, fennel extract was preferred for further studies as compared to green cardamom extracts.

Purification of flavonoids by HPLC

HPLC is a dominant analytical technique used for separation and characterisation of flavonoids. The relatively high molecular mass and intrinsic features of hydrophobic flavonoids aglycones and hydrophilic flavonoids glycosides favour HPLC. HPLC also offers a unique chance to separate simultaneously all components together and even in their low concentrations (Stalikas, 2007). Various standards such as gallic acid, ellagic acid, quercetin, ferulic acid and kaempferol were run using the acetoniitrile: water system. The sample was then run to identify the various flavonoids present in the extract with respect to the standards using the reference of the standards which indicated the presence of ellagic acid and quercetin in the fennel extracts [Figure 2]. Previous studies reveal the presence of gallic acid, caffeic acid, ellagic acid, quercetin and kaempferol in the treated methanolic extracts of fennel (Dua et al., 2013).

Inhibition of Histidine Decarboxylase

The enzyme was isolated and purified by sonication and ammonium sulphate precipitation, ion exchange chromatography, molecular exchange chromatography, respectively. The enzyme activity was calculated after each purification step. The purified enzyme was further subjected to inhibition studies. The incubation of the enzyme with the inhibitor causes binding of the inhibitor to specific sites of the enzyme decreasing its efficiency of binding to the substrate and thus, decreasing the concentration of the product. Overall, a decrease in enzyme activity can be observed. The activity of enzyme in presence of varying concentrations of inhibitor was determined and a reciprocal plot, Dixon plot was plotted to determine the concentration of the inhibitor at which it inhibits the enzyme. With the help of Dixon plots, it was determined that...
fennel inhibited the reaction at concentrations of 0.625 μ moles [Figure 3]. An inhibitor which inhibits reactions at nanomoles concentration is said to be a very good inhibitor whereas the ones inhibiting the reaction at millimoles concentration is said to be a very bad inhibitor. The inhibitory concentrations in our study is found to be at μ moles concentration indicating that it partially inhibits the enzyme and does not cause complete inhibition of the enzyme.

**Determination of Insilico pharmacokinetics and bioactivity**

It is a basic necessity of any hit molecule or lead compound to pass a certain set of criteria in order to be a potential drug molecule. The flavonoids isolated from fennel extracts were studied for their role as inhibitors and they were subjected to SwissADME in order to understand their properties which facilitate them with drug-likeliness. In order to be an effective drug, a potent molecule must reach its target in sufficient concentrations and has to be in the bioactive form for a period long enough to bring about an expected biological event. The assessment of absorption, distribution, metabolism and excretion is important earlier in the process of drug development (Daina et al., 2017). SwissADME provides a free web tool in order to identify the extent of these properties thus, determining the drug-likeliness of a potent molecule. The physicochemical properties give an idea about the molecular weight of the molecule along with its ability to accept and donate hydrogen in order to facilitate the formation of hydrogen bonds. LogP determines the lipophilicity of a given molecule with respect to the partition coefficient between n-octanol and water. A higher logP value indicates greater lipophilicity. Various different computational models were used for its prediction. LogS determines the aqueous solubility of the molecule. A negative value indicates a low water solubility whereas a positive value indicates greater solubility. The physicochemical properties of the molecules are depicted below in the Table 1.

The pharmacokinetics of a molecule include its uptake from site of administration and also its ability to cross certain barriers possessed by the host system. It also includes the metabolism of the molecules by the action of a range of host enzymes which help in biotransformation. The permeability across the membranes of certain tissues holds a key to its bioactivity. In case of oral drugs and parenteral drugs gastrointestinal absorption plays a major role. GI absorption helps the drug to enter the blood stream and reach its target to bring about the required action. At the same time, the molecules permeable to the blood brain barrier possess a threat to the host system as they can produce toxicity due to their entry into the brain. The ability of the molecule to act as substrate to the permeability glycoprotein defines ability of the molecule to appraise active efflux through biological membranes. In order for a molecule to reside inside a cell it shouldn’t be a substrate for Pgp which acts as an ATP driven ABC transporter. Pharmacokinetics also determines the ability of the molecule to interact with a group of isoenzymes belonging to the cytochrome P450 superfamily. These isoenzymes help eliminates drugs from the host body through metabolic transformation. Inhibition of this group of enzymes can cause toxic effects due to lower drug clearance and higher accumulation of drug in the host body. LogKp determines the skin permeability coefficient. A greater negative value indicates lesser skin permeability. This parameter plays an important role for drugs used as topical agents. The pharmacokinetic properties of the flavonoids are listed in Table 2.

Drug likeliness assesses qualitatively the chance of a molecule to become an oral drug with respect to bioavailability. The Lipinski filter is the most important set of rules that are implemented in order to identify the likeliness of a molecule in order to act as a drug. Various other models of drug likeliness have also been adopted by different pharma companies according to their convenience. The Lipinski filter is adopted by Pfizer, the Ghose model is adopted by Amgen, the Veber model by GSK, the Egan model by Pharmacia and the Muegge model by Bayer. The drug likeliness is determined by whether the molecule follows all the set of rules indicated by the model without any violation. Any violation in it renders the molecule ineffective of drug likeliness. The Bioavailability score determines the oral bioavailability of a molecule as it is very necessary for the molecule to be bioavailable in order to bring about the required biological effect. Quercetin turns out to be a very promising molecule following all the different models of drug likeliness whereas ellagic acid fails in the Veber model and Egan model. Both have similar bioavailability scores indicating a very low oral bioavailability. The properties of drug likeliness of flavonoids are depicted in the Table 3.

The medicinal chemistry section helps in drug discovery by identifying fragments that can prove problematic. PAINS (Pan assay interference) identifies compounds or molecules that contain substructures that exhibit potent response irrespective of targets. It identifies fragments that yield a false positive biological output. Brench is a structural alert which identifies potentially toxic, reactive and metabolically unstable molecule which can be responsible
Table 4: Medicinal chemistry of flavonoids

| Compound Name | PAINS          | Medicinal chemistry | Synthetic accessibility |
|---------------|----------------|---------------------|-------------------------|
| Ellagic acid  | Catechol alert | Catechol, Cumarine, PAH alert | Yes | 3.17 |
| Quercetin     | Catechol alert | Yes | 3.23 |

Table 5: Molecular properties of flavonoids in fennel

| Sl. No. | Compound Name | Log P | TPSA  | Molecular weight | Hydrogen bond acceptors | Hydrogen bond donors | Rotatable bonds | Molecular Volume |
|---------|----------------|-------|-------|------------------|------------------------|---------------------|-----------------|------------------|
| 1       | Ellagic acid   | 0.94  | 141.33| 302.19           | 8                      | 4                   | 0               | 221.78           |
| 2       | Quercetin      | 1.68  | 131.35| 302.24           | 7                      | 5                   | 1               | 240.08           |

Table 6: Bioactivity scores of flavonoids in fennel

| Sl. No. | Compound Name | GPCR ligand | Ion channel modulator | Kinase inhibitor | Nuclear receptor ligand | Protease inhibitor | Enzyme inhibitor |
|---------|---------------|-------------|-----------------------|-----------------|------------------------|-------------------|-----------------|
| 1       | Ellagic acid  | -0.29       | -0.27                 | -0.01           | 0.11                   | -0.18             | 0.17            |
| 2       | Quercetin     | -0.06       | -0.19                 | 0.28            | 0.36                   | -0.25             | 0.28            |

for poor pharmacokinetics. Lead likeness determines the suitability of the molecule for optimization which include modifications to increase the lipophilicity and size. Synthetic accessibility determines how easy or difficult is it for a molecule to be synthesized where a score of 1 indicates a very easy synthesis and a score of 10 determines the synthesis of the molecule to be very hard. Both ellagic acid and quercetin exhibit interference due to catechol structures in them and can be optimized as potential leads with an intermediate synthetic accessibility. The properties of flavonoids in relation to medicinal chemistry are depicted in Table 4. [Figures 4 and 5]

Molinspiration, web-based software was used to obtain parameter such as Mi Log P, TPSA, drug likeness scores. Mi Log P is calculated by the methodology developed by Molinspiration as a sum of fragment-based contributions and correction factors. Mi Log P parameter is used to check good permeability across the cell membrane. Partition coefficient or Log P is an important parameter used in rational drug design to measure molecular hydrophobicity. Hydrophilic/lipophilic nature of drug molecule affects drug absorption, bioavailability, drug–receptor interactions, metabolism of molecules, as well as their toxicity. Molecular Polar Surface Area TPSA is calculated based as a sum of fragment contributions of O and N-centered polar fragments. Total polar surface area (TPSA) is closely related to the hydrogen bonding potential of a molecule and is a very good predictor of drug transport properties such as intestinal absorption, bioavailability, blood brain barrier penetration etc.

Calculation of volume developed at Molinspiration is based on group contributors. Number of rotatable bonds measures molecular flexibility. It is a very good descriptor of absorption and bioavailability of drugs. Through drug likeness data of molecule, it can be checked for molecular properties and structure feature in respect to known drugs. Bioactivity of the drug can be checked by calculating the activity score of GPCR ligand, ion channel modulator, nuclear receptor legend, kinase inhibitor, protease inhibitor, enzyme inhibitor. All the parameters were checked with the help of software. Calculated drug likeness score of each compound were compared with the specific activity of other compounds and the results were compared with standard drug. For organic molecules the probability is if the bioactivity score is (> 0), then it is active,
if (-5.0 – 0.0) then moderately active, if ( -5.0) then inactive. The drug likeness scores were calculated by considering Mi Log P (partition coefficient), molecular weight, number of heavy atoms, number of hydrogen donor, number of hydrogen acceptor and number of violations, number of rotatable bonds and volume. Structures of ellagic acid and quercetin were drawn using online molinspiration for calculation of molecular properties and bioactivity score (Anastas and Lankey, 2002); (Thirumurugan et al., 2010); (Thompson and Yates, 2006). The results of these are listed in the Tables 5 and 6.

The properties of the flavonoids determined by Molinspiration indicate that both the molecules i.e., Ellagic acid and quercetin obey the Lipinski’s rules and thus, can be probable lead molecules with drug likeliness. Their bioactivity scores indicate their ability to act as a ligand to the nuclear receptor and also as an enzyme inhibitor which adds a positive note to its function as an inhibitor of the enzyme, histidine decarboxylase. Quercetin has an added property of acting as an inhibitor to the kinase receptor [Figures 6, 7, 8 and 9].

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Conflict of Interest
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