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EVALUATION OF ANTI-*HELICOBACTER PYLORI* (DSMZ 10242) ACTIVITY AND QUALITATIVE ANALYSIS OF QUERCETIN BY HPLC IN *PHYLLANTHUS NIRURI* LINN.

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ABSTRACT

Phyllanthus niruri Linn. is traditionally used in folk medicine for the treatment of a variety of ailments such as asthma, arthritis, poor appetite, constipation, cuts and bruises, corneal opacity, conjunctivitis, flu and colds, blennorrhagia, colic, diabetes, dropsy, dysentery, dyspepsia, fever, flu, gout, gonorrhea, itch, jaundice, kidney aliments, leucorrhea, malaria, menorrhagia, menstrual troubles/complaints, obesity, proctitis, stomachache, tenesmus, tumor, typhoid fever and vaginitis. The present study was aimed to evaluate the anti-*Helicobacter pylori* and urease inhibition activities of hydroalcoholic extracts from different regions of Punjab using simple plate assays. From the study it was reported that hydroalcoholic extract from Patiala exhibited stronger anti-*H. pylori* activity (20 mm) than extracts from other regions of Punjab. Moreover, quercetin was determined in all the extracts using HPLC and maximum concentration of quercetin was also found in extract from Patiala region (574.8 mg/g). Preliminary studies revealed that the inhibitory mechanism may involve quercetin based non-competitive urease inhibition.

KEYWORDS: Anti-*Helicobacter pylori*, *Phyllanthus niruri* Linn., Urease inhibition, Quercetin, HPLC, Anti-microbial activity.

INTRODUCTION

Helicobacter pylori, is a Gram negative, spiral shaped bacterium recognized as class I carcinogen by World Health Organization (WHO).[1] Infections due to *H. pylori* are more prevalent in developing parts of the world due to the poor socioeconomic status and overcrowded conditions.[2] However, this pathogen can persist in an acidic environment of stomach (pH 2) of an individual for life long without causing any infection, only 10-20% population are likely to get infected and 1-2% develop gastric carcinoma.[2,3] Various pharmacological treatments are used for the eradication of *H. pylori* such as antibiotics (amoxicillin, clarithromycin, metronidazole and tetracycline), bismuth salts, H2 blockers and proton pump inhibitors in conjunction with antibiotics (in Dual, Triple or Quadruple therapy).[4] The success of these treatment regimens is associated with various side effects including treatment cost, effectiveness of antibiotics and resistance towards antibiotics.[4,5] Considering these problems there is need to explore new drugs and to develop alternative therapies with improved stability and low toxicity.[6,7]

The use of Traditional Asian Medicine (TAM) to combat *H. pylori* related disorders has been widely studied. *Phyllanthus niruri* Linn. is a small annual herb widely used in traditional Chinese and Ayurvedic medicine for the treatment of various disease such as asthma, arthritis, poor appetite, constipation, cuts and bruises, corneal opacity, conjunctivitis, flu and colds, blennorrhagia, colic, diabetes, dropsy, dysentery, dyspepsia, fever, flu, gout, gonorrhea, itch, jaundice, kidney aliments, leucorrhea, malaria, menorrhagia, menstrual troubles/complaints, obesity, proctitis, stomachache, tenesmus, tumor, typhoid fever and vaginitis. This plant is rich source of bioactive constituents/phytochemicals such as alkaloids, coumarins, flavonoids, lignans, saponins, tannins and terpenoids.[8]

Flavonoids are the primary constituents of herbal medicines and possess anti microbial activity. Flavonoids such as quercetin, rutin, quercitrin, astragalin and catechin have been identified in extracts from *P. niruri*.[9] Out of these flavonoids, quercetin belonging to class flavonols is widely studied bioflavonoid because of its health promoting advantages such as anti-inflammatory, anti-nociceptive, anti-mutagenic, anti-leishmanial and anti-viral activity. It acts as gastroprotective agent and also prevents osteoporosis.[10-16] Quercetin plays a very important role in the prevention and treatment of peptic ulcer by promoting mucus secretion. It has also been shown to inhibit the growth of *H. pylori* in *in-vitro* studies.[17]

The study was undertaken, keeping in mind the need to identify the potential of this medicinal plant against *H. pylori*. To the best of our knowledge hydroalcoholic extracts of *P. niruri* haven't been screened yet for their anti-*H. pylori* activities. The extract exhibiting promising anti-*H. pylori* activity was furthur tested for the presence of quercetin, as it seems to play an important role in inhibition and prevention of colonization by *H. pylori* in stomach.

MATERIALS AND METHODS

Collection of Plant material

Different populations of *Phyllanthus niruri* were collected from various regions of Punjab, India viz. Bathinda (Krishi Vigyan Kendra, Kheti Bhawan, Rose Garden and Chetak Park), Amritsar (Guru Nanak Dev University, Khalsa College), Roopnagar (Chamkaur Sahib, Bhakra Nangal dam and Gurudwara Sadabarat) and Patiala (Punjabi University, Baradari Garden and Urban Estate). The plant samples were identified by Botanist Dr. Geetika Sirhindi, Department of Botany, Punjabi University, Patiala. The collected populations were washed thorougly under running water to remove soil and other extraneous matter. The samples were then shade dried properly for atleast a month and crushed using electric grinder to get fine powder.

Preparation of Hydroalcoholic Extracts

Air dried plant powder of *P. niruri* was minced and extracted with 50% ethanol and water in 1:3 ratio. The fraction was then macerated at room temperature (25±3°C) for 15 days. The solvent was evaporated and the extract was concentrated to desired level using vaccum evaporator and stored at -20°C until furthur analysis.[18]

Procurement and Maintenance of microbial culture:

H. pylori (DSMZ) 10242 was procured from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Germany. It was subcultured and maintained using Brain Heart Infusion (BHI) media supplemented with 5-10% blood (HiMedia) at 37°C in 5% CO_2 atmosphere throughout the study as described by DSMZ. The culture used was maintained as glycerol stock and stored at −20°C for further utilization. The work was carried out using class II biosafety cabinet at Department of Clinical Microbiology, PGIMER (Post Graduate Institute of Medical Education and Research), Chandigarh under Dr. Vikas Gautam.

Antibacterial Assays of Plant extracts

The crude extracts were filtered through HEPA filters (0.47 micron membrane filter) and the filterates were further used for *in vitro* testing of extracts using agar well diffusion assay and disc diffusion assay. Each experiment was repeated thrice and the control consisted of 50% ethanol and water in ratio 1:3 in each antimicrobial assay. The lowest concentration of each of the extract which inhibited the bacterial growth was observed in triplicate analysis.

a) Well diffusion assay

For this assay, method of Divakar and co-workers[19] was followed along with some modifications. Around 6 mm diameter wells were made using sterile cork borer on BHI agar media having 5-10% v/v blood, overlaid with 5 ml soft BHI agar (0.8%) containing *H. pylori* and each well was inoculated with 5-50 µl plant extracts. The plates were incubated for 48 hrs at 37°C in 5% v/v CO_2 and inhibition zones around wells were measured. Inhibition was scored positive if the zone was wider than 2 mm in diameter.

b) Disc diffusion assay

Antibacterial susceptibility using disc diffusion assay was assessed by the method of Yildrim and Jhonson[20] with modifications. The filtered plant extracts (5-50 µl) were spotted on sterile discs 8 mm in diameter on preseeded BHI agar plates containing 5-10% v/v blood. Plates were preseeded with *H. pylori* using sterile swab, which was dipped into the respective microbial suspensions and surplus removed by rotation of the swab against the sides of the tube above the fluid level. Plates were incubated at 37°C for 48 hrs under microaerophilic environment i.e. 5% v/v CO_2. After incubation the plates were observed for inhibition zones around the discs.

Urease Inhibition Assay

Urease plate assay is based on the principle that either urease is located in the cytoplasmic membrane or excreted by the bacteria under low-pH conditions, will convert urea to ammonia and counter the low pH. Under these circumstances, due to rise in pH, bromocresol purple will be converted to purple color. Thus, due to inhibition of urease activity, a yellow zone would be observed. For this, urea (10mM) was added to the BHI medium containing agar (3% w/v) supplemented with 5-10% v/v blood and bromocresol purple (0.01 g/liter). The final pH of the medium was adjusted to 6.0. The BHI media was overlaid with *H. pylori* using sterile swab as described earlier. Individual hydroalcoholic extracts from different regions were added to sterile paper disks using a micropipette. Saturated disks (5-50 µl) were

stored in dark at -20°C until use. BHI agar plates with *H. pylori* were spotted with saturated discs and plates were incubated at 37°C for 48 h in CO_2 incubator with 5% v/v CO_2. The plates were observed for the presence of yellow zones and diameter of each zone was recorded.[21]

HPLC Analysis of Extracts for the Presence of Quercetin

The filtered hydroalcoholic extracts from different regions along with authentic quercetin sample (1000 µg/ml) were injected in HPLC system equipped with PDA (Photo Diode array) detector. Column was a Dynamax C18, with 250 x 4.6 mm dimensions and 5 µm particle size. Qualitative analysis was made, in step gradient mode, with water, acetonitrile and acetic acid as mobile phase in ratio 90:10: 0.2 at a flow-rate of 1 mL min^{-1}. The injection volume was 20 µL and the eluate was monitored at 339 nm.[22, 23] Percentage quercetin in each extract was calculated using formula.[24]

% Quercetin= Area of test/Area of sample*100.

Conversion factor: 1 per = 10mg/g.

Statistical analysis

All experiments were performed in triplicates and the results are expressed as mean ± standard deviation.

RESULTS

Antibacterial Assays of Plant extracts

The antibacterial activity of *P. niruri*, hydroalcoholic extracts from various regions of Punjab was tested against *H. pylori* DSMZ 10242. Extracts were first diluted to different ratios ranging from (1:99-1:999). However, none of the dilution inhibited growth of *H. pylori*. After several attempts, the whole extracts were finally used for antibacterial assays as described before without any dilution at different concentrations (5-50 µl).

a) Agar well diffusion assay

The hydroalcoholic extracts from different regions showed inhibitory activity against *H. pylori* at concentration of 50 µl (Table 1 and Fig. 1). However, maximum inhibition was shown by hydroalcoholic extract from Patiala region (13 mm), followed by Roopnagar (10 mm) and Amritsar (8 mm). Lowest inhibition was recorded in hydroalcoholic extract from Bathinda (6mm).

b) Disc diffusion assay

In this assay similar inhibition pattern was recorded as in agar well diffusion aasay (Table 1 and Fig. 1). Maximum inhibition was recorded in case of hydroalcoholic extract from Patiala region (20 mm), followed by Roopnagar (15 mm) and Amritsar (10 mm). Again least inhibition zone was shown by hydroalcoholic extract from Bathinda (5 mm).

Urease Inhibition Assay

Inhibitory effect of extracts over urease was tested and it was found that the hydroalcoholic extracts from all the four regions inhibited urease. When the pH of the plates was adjusted to 6.0, the color of medium changed from purple to yellow. After inoculation as the bacteria multiplied in number, the color of medium changed from yellow to purple by producing ammonia through urease activity and thus countering the effect of pH in the medium. However, the area around the paper disks didn't change to purple indicating that urease was inhibited by the extracts. Larger the yellow zone, higher the inhibition by the extracts. The hydroalcoholic extract from Patiala showed most potent inhibitory effect against urease by giving maximum zone of inhibition i.e. 20 mm, followed by Roopnagar (14 mm), Amritsar (8 mm) and Bathinda (6 mm) (Table 1 and Fig. 1).

Table 1: Anti-*H. pylori* activity of Hydroalcoholic extracts of *Phyllanthus niruri* from different regions of Punjab.

S. No.	Hydroalcoholic Extracts (Regions)	Quercetin content (mg/g)	Zone of inhibition (mm)		
			Well Diffusion Assay	Disc Diffusion Assay	Urease Inhibition Assay
1.	Amritsar	0	8.0±0.71	10.0±0.75	10.0±0.78
2.	Bathinda	0	6.0±0.86	5.0±0.78	8.0±0.78
4.	***Patiala***	***574.8***	***13.0±0.74***	***20.0±0.62***	***20.0±0.83***
3.	Roopnagar	393	10.0±0.98	15.0±0.70	14.0±0.92

* Results are mean ± S.D.

Figure 1: Anti-*H. pylori* and urease inhibition aasay of *Phyllanthus niruri* Linn. from Punjab where, A; Amritsar, B; Bathinda, P; Patiala, R; Roopnagar and C; Control.

HPLC Analysis of Extract from Patiala for the Presence of Quercetin

The hydroalcoholic extracts from various regions were also tested for the presence of flavonoid quercetin. The method provided a quick analysis of the hydroalcoholic extract. The conditions used provided good separation of the peaks which were identified in the chromatogram (Fig. 2) as quercetin (Rt= 5.32) in hydroalcoholic extract from Patiala and (Rt= 5.23) in Roopnagar hydroalcoholic extract, by comparing them with the chromatogram of the quercetin reference compound (Rt=5.3) (Fig. 2) obtained under same conditions on line. However, in hydroalcoholics extracts of Amritsar and Bathinda region, quercetin was not detected. Area percentage was calculated in all the four extracts and maximum area percentage was found in extract from Patiala (574.8%) followed by Roopnagar (393%) (Table 1). Thus, it was postulated that the inhibition activity of the extracts may be due to the presence of quercetin.

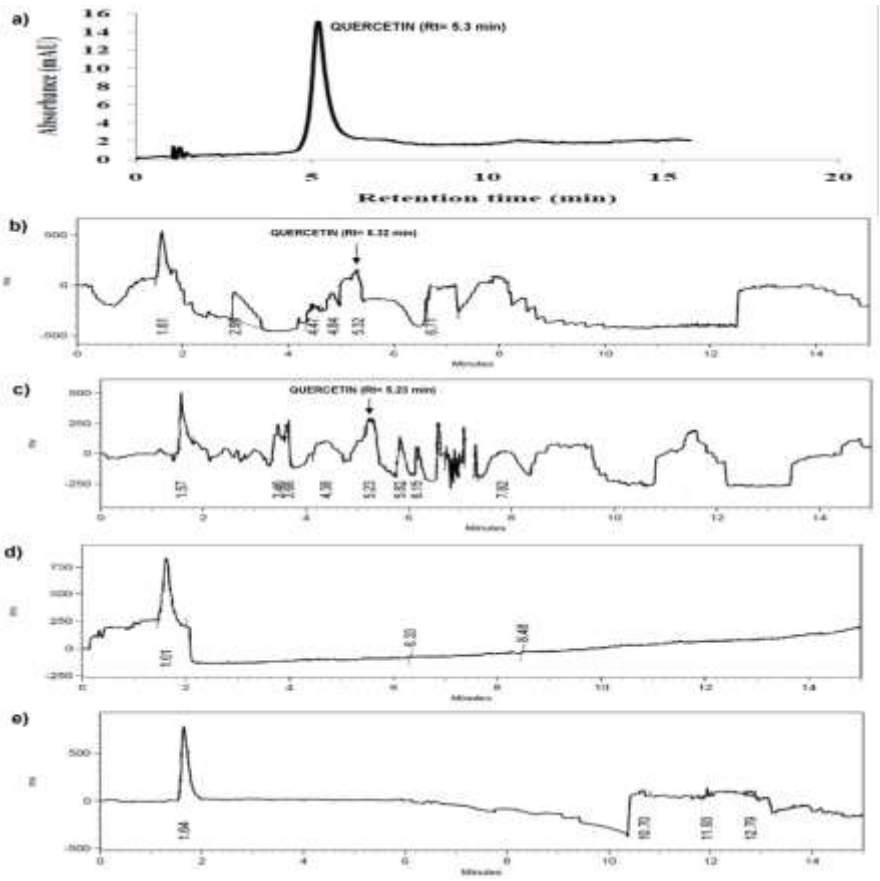

Figure 2: HPLC Fingerprinting of Hydroalcoholic extracts of different P. niruri populations from Punjab a) Authentic Quercetin standard b) Patiala c) Roopnagar d) Amritsar e) Bathinda. Column: Dynamax C18 (250 x 4.6 mm, 5 µm); Eluent: Water: Acetonitrile: Acetic acid, 90:10:1 (0-15 min); Flow-rate: 1 mL min-1; Detection wavelength: 339 nm.

Table 2: Plants tested for anti-*H. pylori* activity along with their mechanism of action.

S.No.	Plant	Plant Part	Extract used	Mechanism of Action	References
1.	*Acacia nilotica*	Leaves and flowers	Aqueous	Urease inhibition	[6]
2.	*Achillea millefolium*	Leaves/Root/ Stem	Ethanol/ Methanol	Urease inhibition	[26]
3	*Acorus calanus*	Root	Methanol/water	Urease inhibition	[30]
4.	*Adhatoda vasica*	Whole plant	Acetone and Methanol	Urease inhibition	[6]
5.	*Adhatoda zeylanica*	Root/ Stem	Ethanol	Urease inhibition	[26]
6.	*Allium sativum*	Dried fruits	Water	Reduced IL-8 expression in infected AGS cells	[31]
7.	*Angelica archangelica*	Leaf	Methanol/water	Urease inhibition	[30]
8.	*Aristolachia bracteata*	Leaves/Stem	Ethanol/ Methanol	Urease inhibition	[26]
9.	*Artemisia scoparia*	Whole plant	Methanol	Urease inhibition	[32]
10.	*Brassica oleracea*	Sprouts	-	Sulphoraphane increase mammalian cytoprotective response	[3]
11.	*Calophyllum brasiliense*	Stem bark	Hydroethanol/ dichloromethane	Urease inhibition and modulation of endogenous antioxidant system	[33]
12.	*Calotropis procera*	Leaves and flowers	Aqueous and acetone	Urease inhibition	[6]
13.	*Camellia sinesis*	Leaf	Ethanol	Urease inhibition	[34]
14.	*Cassia obtusifolia*	Whole plant	Ethanol	Urease inhibition	[35]
15.	*Casuarina equisetifolia*	Fruit	Methanol	Urease inhibition	[6]
16.	*Cinnamom*	Powder	Ethanol	Urease inhibition	[36]
17.	*Citrus arantifolia*	Fruit	Methanol/water	Urease inhibition	[34]
18.	*Cuminum cyminum*	Dried fruits	Water	Reduced IL-8 expression in infected AGS cells	[31]
19.	*Cuscuta reflexa*	Stem	Ethanol	Urease inhibition	[26]
20.	*Eucalyptus globules*	Leaves	Ethanol	Urease inhibition	[26]
21.	*Fagonia arabica*	Whole plant	Acetone	Urease inhibition	[6]
22.	*Lauras nobilis*	Leaf	Methanol/water	Urease inhibition	[30]
23.	*Malva parviflora*	Leaves/Root/ Stem	Ethanol	Urease inhibition	[26]
24.	*Mangolia officinalis*	whole plant	Ethanol	Urease inhibition	[35]
25.	*Matricaria recutita*	Flower	Methanol/water	Urease inhibition	[34]
26.	*Mentha*	Leaves/Root/	Ethanol/ Methanol	Urease inhibition	[26]

	longifolia	Stem			
27.	*Nasturtium officinale*	Leaf	Methanol/water	Urease inhibition	[34]
28.	*Nigella sativa*	seed	Methanol/water	Urease inhibition	[30]
29.	*Origanum vulgare*	Whole plant	Water	Inhibition of proline dehydrogenase and urease activity	[21]
30.	*Pelargonium sidedoides (EPs) 7630*	Roots	Ethanol	Inhibit adhesion on stomach cells	[37]
31.	*Piper nigrum*	Dried fruits	Water	Reduced IL-8 expression in infected AGS cells	[31]
32.	*Pistacia lentiscus*	Bark	Mastic gum	Reduce colonization	[1]
33.	*Phyllanthus niruri*	Whole plant	Hydroalcoholic	Inhibition of urease by quercetin	Present study
34.	*Prunus mume*	Fruit	Fruit juice concentrate	Inhibition of urease and inhibition of motility by syringaresinol	[38]
35.	*Punica granatum*	Flower	Methanol/water	Urease inhibition	[34]
36.	*Vaccinium macrocarpon*	whole plant	Water	Inhibition of proline dehydrogenase and urease activity	[21]
37.	*Zingiber officinale*	Rhizome root	Methanol/water	Urease inhibition	[30]

Table 3: Characterized phytochemicals exhibiting urease inhibitory activity[4,37,40,41]

S. No.	Phytochemical	Plant	IC50 value
1.	(E)-2-haxenel	*Annacardium occidantale*	50g/ml
2.	1,2,3,4,5,6-penta-O-galloyl-D-glucoside	*Paeonia lactiflora*	72M
3.	Allian diallyl thiosulphinate	Garlic	-
4.	Anacardic acid	*Annacardium occidantale*	125 /ml
5.	Atranorin	*Sterospermum acuminatissimum*	18.2µM
6.	Baicalin	*Scutellariae baicalensis*	2.7mM
7.	Caseadine	*Corydalis govaniana*	20.2µM
8.	Caseamine	*Corydalis govaniana*	66.7µM
9.	Decarboxymethyl ligstroside aglycone	Olive oil	1.3µg/ml
10.	Genstein	*Pueraria thunbergiana*	430/ml
11.	Govaniadine	*Corydalis govaniana*	38.9µM
12.	Juglone	*Juglans nigra*	4.8µM
13.	Luteolin	*Lonicera japonica*	35.5µM
14.	Luteolin-7-O-glucoside	*Lonicera japonica*	55.8µM
15.	Methyl gallate	*Paeonia lactiflora*	1.3mM
16.	Myricetin	*Lonicera japonica*	77.2µM
17.	Myricitrin	*Lonicera japonica*	98.7µM
18.	Ophiamide A	*Heliotropium ophioglossum*	23.1µM
19.	Ophiamide B	*Heliotropium*	12.6µM

		ophioglossum	
20.	Patchouli alcohol	*Pogostemon cablin*	77g/ml
21.	Protopine	*Corydalis govaniana*	54.1µM
22.	Quercetin	*Lonicera japonica*	11.2µM
23.	Quercetin-4'-O-D-glucoside	*Allium cepa*	190
24.	Rutin	*Lonicera japonica*	67.6µM
25.	Scutellarin	*Erigeron beriscapus*	1.3mM
26.	Shoreaphenol	*Hopea exalata*	126.8µM
27.	Sulphoraphane	*Brassica oleracea*	-
28.	Tectorigenin	*Pueraria thunbergiana*	0.43mg/ml
29.	Vernonione	*Vernonia cinevascens*	227.6µM
30.	Zygophyloside A	*Zygophyllum fabago*	500
31.	Zygophyloside E	*Zygophyllum fabago*	500
32.	Zygophyloside G	*Zygophyllum fabago*	500 µM

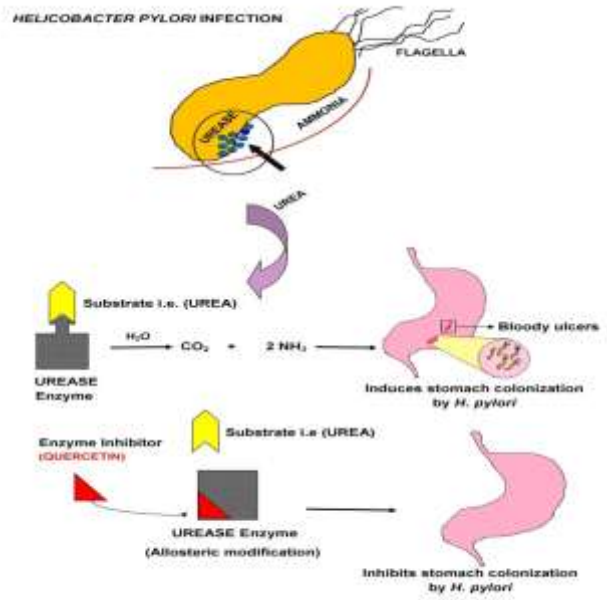

Figure 3: Non-competitive inhibition of enzyme urease by quercetin.

DISCUSSION

Due to therapeutic failure of some antibiotic resistant *H. pylori* strains, research has been shifted to potential use of plant secondary metabolites/ phytochemicals as new anti-ulcer drugs. Phytotherapy has recently gained significant attention due to less side effects and effective disease control. Plants exhibiting anti-*H. pylori* activity along with their mechanism of action is given in table 2.

In the present study the data revealed that hydroalcoholic extracts from all the four regions of Punjab has significant inhibitory effect against *H. pylori*. In a previous study, aqueous extract of *P. niruri* from Ecuador and Peru has been tested by Ranilla and co-workers [9] for anti-*H.*

pylori activity and it was found that both the extracts inhibited *H. pylori* ATCC 43579 in dose dependent manner. The inhibitory effect was accredited to the presence of ellagitannins, geraniin or corilagin, previously identified in the leaves of *P. niruri*. The study postulated that these ellagitannins were partially hydrolysed after the hot water extraction following the formation of ellagic acid, gallic acid and other ellagitannin derivatives. Similarly, *Phyllanthus urinaria* was studied earlier for anti-*H. pylori* activity in AGS cells (Human gastric epethelial cells) and found to inhibit invasion and adhesion by *H. pylori*. The extract also inhibited *H. pylori*-induced nuclear factor (NF)-kappa B activation, and the subsequent release of interleukin (IL)-8 in AGS cells. The chemopreventive activity was accredited to group of flavanoids namely β-sitosterol-3-O-β-d-glucopyranoside, methyl gallate, methyl brevifolincarboxylate, rhamnocitrin, rutin, trimethyl-3,4-dehydrochebulate, phyllanthin, phyltetralin and quercitrin.[25]

Urease enzyme is considered as the main virulence factor of *H. pylori*. It hydrolyzes urea into ammonia, thereby creating a friendly environment for the survival of this gut pathogen.[26] Many urease inhibitors have been described earlier such as fluorofamide, hydroxyurease and hydroxamic acids etc. but their use has been prohibited due to toxicity and instability. Active phytochemicals from medicinal plants with urease inhibitory activity are less toxic potential therapeutics with higher stability (Table 3). In the present study inhibition of *H. pylori* urease by extracts of *P. niruri* from various regions of Punjab were tested using simple plate assay. The hydroalcoholic extracts of *P. niruri* from different regions of Punjab were found to exhibit urease inhibition activity and maximum zone of inhibition i.e. 20 mm was shown by extract from Patiala region. HPLC of extracts was further performed to find active principle behind inhibition and quercetin was regarded as the reason behind it.

The results are supported by the study of Xiao et al.[27], they tested 20 flavonoids, out of which quercetin showed maximum potency with IC50 of 11.2+/- 0.9 mu M against urease from *H. pylori* ATCC 43504. The research group suggested quercetin as non-competitive urease inhibitor. Moreover, 3-, 5-OH as well as 3', 4'-dihydroxyl groups were regarded as key structural components for active compounds and removal of these groups led to a sharp decrease in inhibition activity of quercetin. The diagramatic representation of inhibition of urease activity by quercetin is given in Fig 3.

In another study Shin et al.[28] studied the effect of quercetin on the growth and vacuolation of *H. pylori*. The results suggested that quercetin inhibited *H. pylori* Vac A vacuolation in HeLa

cells with IC50 value of 0.046 mM. vac A gene encodes Vac A protein which is strongly associated with damage of epethelial lining of gastric tissue and gastric ulcers.[29] It also inhibited procaspase-3 activation to caspase-3 induced by *H. pylori* Vac A toxin and resulted in induction of cell death by activating a cascade of proteolytic caspases.

Quercetin is a dietary flavonoid possessing strong antioxidant properties. However, *in vivo* studies on the safety and efficacy of *P. niruri* extracts are extremely important in our ongoing research work to formulate new herbal drug and make it available for further clinical use. Currently the intake of plants containing quercetin is recommended to prevent incidents of gastric ulcers caused due to *H. pylori*.

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