THE ANTIOXIDANT AND H^+K^+ ATPASE INHIBITORY EFFECT OF *ANDROGRAPHIS PANICULATA* AND ANDROGRAPHOLIDE –*IN VITRO* AND *IN VIVO* STUDIES

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Summary

Andrographis paniculata popularly known as kalmegh of ayurveda and the andrographolide present in the leaves have been widely included in various drug formulations used for liver and gastric ailments. The aim of the present study was to evaluate the *in vitro* antioxidant and the antiulcer activities of the extracts of aerial parts of *Andrographis paniculata* prepared by using solvents such as petroleum ether, ethyl acetate, ethanol, hydroalcohol and of purified andrographolide. The antiulcer effect was assessed by the capacity to inhibit the H^+K^+ ATPase activity of sheep gastric mucosal cells *invitro*. The antiulcer activity was also evaluated *invivo* with different experimental ulcer models in rats. The antioxidant activity was determined by using DPPH, superoxide, hydroxyl and nitric oxide generating models and by reducing power. The HPLC-UV chromatogram analysis revealed the presence of important constituents such as Quercetin and Formononetin. Among all the fractions studied, hydroalcoholic fraction exhibited significant free radical scavenging activity in all the models. The activities were found to be dose dependent and this study indicates that both hydroalcoholic extract and andrographolide exhibit free radical scavenging activity *in vitro*. The test compounds were found to suppress sheep mucosal H^+K^+ ATPase activity *invitro* and the ulcer score *invivo*. So further study is needed to confirm the gastroprotective property of *Androraphis paniculata.*

Key words: *Androraphis paniculata;* andrographolide; free radicals; scavenging activity; H^+K^+ATPase; antiulcer property.

Introduction

Andrographis paniculata also known as king of bitters is the member of plant family Acanthaceae and has been used for centuries in Asia to treat gastrointestinal discomfort, liver disorders and a variety of other chronic and infectious diseases. It is included in twenty six ayurvedic formulations shown in ancient Indian pharmacopoeia. The abortifacient, acrid, analgesic, anti-inflammatory [1, 2], antibacterial, antiviral [3], antipyretic [4, 5], hypoglycemic, cardioprotective, hepatoprotective and immune enhancement properties of *Andrographis paniculata* have shown that the plant has a broad range of pharmacological effects.

Andrographolide, a chief constituent of the leaves is a bitter water soluble lactone exhibiting protective effect on CCl_4 induced hepatopathy in rats. Earlier work showed that andrographolide has multiple pharmacological acivities such as inhibition of platelet activation [2], stimulation of cell differentiation [6] and stimulation of immunity. Chiou *et al* [7, 8] reported that andrographolide inhibited nitric oxide production in macrophage cell line.

Peptic ulceration is a common, chronic, recurrent, and occasionally life- threatening disease of poorly defined pathogenesis in humans [9, 10]. The gastric mucosa is continuously exposed to potent injurious agents such as acid, pepsin, bile acids, food ingredients, bacterial products and drugs [11]. Increase in gastric acid and pepsin secretion, decrease in gastric blood flow, suppression of endogenous generation of prostaglandins, inhibition of mucosal growth and cell proliferation, and alteration of gastric motility have been implicated in the pathogenesis of gastric ulcer [12]. Hyperactivity of H^+K^+ ATPase has been claimed for the excess acidity and mucosal inflammation in gastric ulcer. Hence agents which suppress the activity of H^+K^+ ATPase activity are considered as a choice of antiulcer drugs.

Reactive oxygen species are generated by cells in some physiological and pathological circumstances. Any derangement between in ratio of pro-oxidants to antioxidants, in which pro-oxidants prevail leads to oxidative stress [13]. Insufficient antioxidant protection or excess production of reactive oxygen species can result this condition. Reactive oxygen species can react with all macromolecules such as lipids, proteins, nucleic acids and carbohydrates, particularly polyunsaturated fatty acids of cell membranes. Initial reaction with reactive oxygen species causes a continuous chain reaction resulting in cell injury and ultimately cell death [14]. Peptic ulcer is produced by the imbalance between gastroduodenal mucosal defense mechanism and offensive factors. Some studies revealed that ROS and lipid peroxidation are implicated in the pathogenesis of ethanol induced gastric lesions and gastrointestinal damage [15-17].

Reactive oxygen species produced *in vivo* including superoxide radical (O^-_2), hydrogen peroxide (H_2O_2) and hypochlorus acid (HOCl). H_2O_2 and O^-_2 can interact in the presence of certain transition metal ions to yield a highly reactive oxidizing species, the hydroxyl radical ($^{\cdot}OH$). [18]. Free radicals are chemical species of atoms or molecules that possess an unpaired electron on their outermost orbit. These free radicals are highly unstable and can react with other molecules by giving out or accepting single electron. Although the human body continuously produces free radicals it possesses several defense systems constituting enzymes and radical scavengers [19]. Therefore antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of diseases in which oxidants or free radicals are involved [20].

Active components of plants such as flavonoids, triterpenes and tannins may be regarded as possible phytochemicals against gastric lesions by acting as protective factors or antioxidants. It is believed that the antioxidant activity of polyphenols is an important factor because reactive oxygen and free radicals are related to the occurrence of ulcers.

Herbal medicines are now widely used by nearly 50% of the world population, in a number of instances for the treatment or prevention of digestive disorders [21, 22]. In traditional medicine, numerous plants and herbs are used to treat gastrointestinal disorders. There has been renewed interest in identifying new anti ulcer drugs from natural sources [23].

Many plants such as *Cedrus libani, Centaurea Solstitialis ssp. Solstitialis, Cistus laurifolius, Hypericum scabrum, Plantago major, Sambuous ebulus* and *Spartium junceum* [24] have been proved to exhibit anti-ulcer properties in experimental animals. These plants and their products are proved gastroprotective and found to act by different mechanisms. The ever- increasing problem of ulcer due to advancement in food pattern and life style, demands the identification and evaluation of new plants with anti-ulcer and antioxidant properties. In the present investigation, various fractions of *Andrographis paniculata* and andrographolide were tested for free radical scavenging and H^+K^+ ATPase inhibiting activity. The effect was further confirmed by testing the drug in ulcer induced rats.

Materials and Methods

Plant collection and identification

The plant was purchased from the local market in Chennai and authenticated by Dr. Jayaraman, Taxonomist, Plant Anatomy Research Centre, Chennai (Voucher No. of the Specimen: PARC/ 2008/ 185). The aerial parts were washed, air dried and pulverized to powder form.

Preparation of plant extracts

The test material was subjected to serial extraction with solvents of different polarity in the order of petroleum ether, ethyl acetate, ethanol and hydroalcohol. Briefly, 15-20g of the dried and homogenous powder of *Andrographis paniculata* was soaked in 100ml of petroleum ether 60-80ºC by cold percolation method and the extract was filtered. The residue was air dried and used for subsequent extraction with solvents like ethyl acetate, ethanol, and hydroalcoholic extract was prepared separately in a similar manner. The filtrate collected in each extraction was evaporated in a desiccator and the filtrate was concentrated. The concentrate was lyophilized and used for the study.

Isolation of Andrographolide

The dried leaves of *Andrographis paniculata* were macerated in methonal (4.01x2 times) and kept at room temperature for 3 days. After filtration, the methanol solution was evaporated under reduced pressure. The residue was partitioned between ethyl acetate and water. The water soluble portion was extracted with n-butanol and filtered. The crude andrographolide, a part of which was chromatographed on a silica gel column using chloroform/methanol (20/1) as a solvent to yield pure andrographolide [25].

Antioxidant activity

DPPH radical scavenging activity

The DPPH radical scavenging capacity of the extracts determined by the method described by Viturro *et al*, 1999 [26]. 0.1ml of of extracts (5-25µg/ml) was mixed with 0.1ml of 1, 1'-diphenyl 1-2-dipicryl hydrazine (DPPH) in dimethyl sulphoxide (DMSO) (0.15%) and incubated for 10 min. The absorbance was then read at 450nm in ELISA strip reader (Qualigens). Percentage inhibition was determined and compared with that of similar concentration of ascorbic acid. IC_{50} values were calculated for both test extracts and the standard.

Nitric oxide scavenging activity

The method of Sreejayan Rao 1997 [27] was used to measure the nitric oxide scavenging of the extracts. Various concentrations of the test material (30-150 µg/ml) was dissolved in phosphate buffer (0.025M, pH 7.4) and incubated with sodium nitroprusside (5 µM) in standard phosphate buffer at 25ºC for 5 hrs. After incubation, 0.5ml of the reaction mixture was added with 0.5ml of Griess reagent (equal volumes of 1% sulphanilamide in 2% phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride in water). The absorbance of the Chromophore formed was read at 540nm. The activity was compared with that of rutin.

Superoxide anion scavenging activity

The method of Nishmiki *et al* 1972 [28] was used to measure the superoxide anion scavenging activity of the extracts. Briefly 1ml of nitro blue tetrazolium (NBT) (156 µM NBT in 100mM phosphate buffer, pH 7.4, 1ml of nicotinamide adenine dinucleotide (NADH) in 100mM phosphate buffer, pH 7.4) were added to the 0.1ml of the test extract with varying concentrations (5-25µg/ml). The reaction was started by adding 100µl of phenazonium methosulphate (PMS) (60 µm in 100mM phosphate buffer, pH 7.4) and the reaction mixture was incubated at 25ºC for 5 min. The decrease in absorbance was measured at 560nm against water blank. Curcumin was used as the positive control.

Hydroxyl radical scavenging activity

The assay was based on the method of Halliwell *et al.,* 1987 [29]. 1ml of the reaction mixture contained 100µl of 28mM 2-deoxy-2-ribose in phosphate buffer, pH 7.4, 500 µl of various concentrations of plant extract (10-80µg/ml), 200µl of 200µM ferric chloride and 1.04mM ethylenediamine tetra acetic acid (EDTA). The reaction mixture was incubated and deoxyribose degradation was measured by the thiobarbituric acid (TBA) reaction [30]. The absorbance was measured at 532 nm. Vitamin E was used as the standard.

Reducing Power

Varying concentrations of different extracts (100-1000µg) were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6), 2.5ml of 1% potassium ferricyanide and incubated at 50ºC for 20 min. 1.5 ml of 10% trichloroacetic acid (TCA) was then added to the reaction mixture and the contents were centrifuged at 3000rpm for 10 min. 0.5 ml of the supernatant was collected and mixed with 1 ml of distilled water and 0.5 ml 0.1% ferric chloride. Control was processed similarly with distilled water. Ascorbic acid was used as standard. Increase in absorbance at 700nm indicates increased reducing power [31].

Preparation of Parietal cells

Proton potassium ATPase was prepared from mucosal scrapings of sheep stomach obtained from slaughter house and then homogenized in 200mM Tris-HCl buffer, pH 7.4, centrifuged for 10 mins at 5000xg. The resulting supernatant was subsequently centrifuged at 5000xg for 20 min [32]. The protein concentration in the supernatant was determined with bovine serum albumin as standard [33]. The parietal cell extract was then employed to determine H^+K^+ ATPase activity.

Determination of H^+K^+ ATPase

The H^+K^+ ATPase activity in the presence of different concentrations of test extracts and omeprazole was assayed by the method of Reyes- Chilpa *et al* 2006 [34]. The enzyme source was preincubated with different concentration of the test material (10-80µg) for 30min. The assay was conducted in a mixture contained an aliquot of *Andrographis paniculata* treated enzyme in 20mM tris-HCl, pH 7.4, 2mM magnesium chloride ($MgCl_2$) and 2mM potassium chloride (KCl). The reaction was started with the addition of 2mM adenosine-5'-triphosphate (ATP) and incubated for 30 mins at 30ºC and terminated by the addition of 10% trichloroacetic acid followed by centrifugation at 2000xg. The amount of inorganic phosphorous released from adenosine-5'-triphosphate (ATP) was determined spectrophotometrically at 640nm. The enzyme source was also treated similarly with the standard drug omeprazole and the enzyme activity was measured.

HPLC analysis

A flavonoids profile assay was performed using HPLC-UV chromatogram. It was used at 40°C with linear gradient mobile phase (methanol, water and phosphoric acid) in the ratio of 100:100:1 and solvent mixture (alcohol, water and hydrochloric acid) in the ratio 50:20:8 with flow rate set of 1.5/min. Equal volumes of standard (20µl) and test solutions were injected into chromatograph. The major peaks were determined according to the retention times obtained from authentic standards run at identical conditions.

***Invivo* antiulcer study**

Animals

Male albino Wistar rats (120-140g) were obtained from Kings Institute, Chennai, India. They were acclimatized to animal house conditions, fed commercial pelleted rat chow (Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. Animals were maintained according to the rules and regulations laid down by the Institutional Ethics Committee.

Treatment protocol for antiulcer activity

Rats were divided into VII groups of six animals each.

Group I (a-c) - Rats treated with ulcerogenic agents.
Group II (a-c) -Rats pretreated with 200mg/kg b wt of petroleum ether extract of *Andrographis paniculata* for 30days and then subjected to ulcer induction.
Group III (a-c)-Rats pretreated with 200mg/kg b wt of ethyl acetate extract of *Andrographis paniculata* for 30days and then subjected to ulcer induction.
Group IV (a-c)- Rats pretreated with 200mg/kg b wt of ethanol extract of *Andrographis paniculata* for 30days and then subjected to ulcer induction.
Group V (a-c) -Rats pretreated with 200mg/kg b wt of hydroalcoholic extract of *Andrographis paniculata* for 30 days and then subjected to ulcer induction.
Group VI (a-c)- Rats pretreated with 3mg/kg b wt of andrographolide for 30 days and then subjected to ulcer induction.
Group VII (a-c)- Rats pretreated with 30mg/kg b wt of omeprazole for 30 days and then subjected to ulcer induction.

Ulcer induction

By ethanol (a)

Gastric ulcer was produced by administering ethanol orally 1ml/200g to rats after 12hr fasting. The animals were sacrificed after 1hr of ethanol administration [35].

By aspirin (b)

Aspirin was administered at the dose of 200mg/kg b.wt and ulcer score was determined after 4h. The stomach was cut open along the greater curvature and ulcer index was scored by a person unaware of the experiment protocol in the glandular portion of the stomach [36].

By pylorus ligation (c)

Drugs were administered for a period of 30 days. On day 30, after the last dose, the rats were kept for 18h fasting. Rats were anaesthetized using diethyl ether, the abdomen was opened and pylorus ligation was done without causing any damage to its blood supply. After replacing the stomach carefully the abdomen wall was closed in two layers with interrupted sutures. The animals were deprived of water during the postoperative period [37]. After 4h, stomach was dissected out and the contents collected.

Determination of ulcer score

The ulcer index of gastric mucosal lesions was evaluated by the score system reported by Nie et al., 2003 [38]. Briefly, after collecting gastric juice, the stomach was opened along the greater curvature and rinsed with 0.1mol/L ice-cold PBS. The stomach was then examined under microscope (100x) to observe erosions and made scores as 1-5: 1. small round hemorrhagic erosion, 2. haemorrhagic erosion<1mm, 3. Hemorrhagic erosion = 1-2mm, hemorrhagic erosion = 2-3mm, 5. haemorrhagic erosion>4mm. The score was multiplied by 2 when the width of the erosion is larger than 1mm.

Statistical analysis

Data were analyzed by using a commercially available statistics software package (SPSS for window V.7.5). Student's t test was performed and results were presented as mean ± S.E.M.

Results & Discussion

Reactive oxygen species and other free radicals have been implicated in the pathology of many disorders like neurodegenerative diseases, inflammation, cataract, liver cirrhosis and gastrointestinal disorders including ulcer [39]. Herbal drugs containing radical scavengers are widely applied for the prevention and treatment of various diseases. Larson [40] reported that phenolic compounds, flavanoids and tannins are the major constituents in most of the medicinal plants to possess antioxidant activity and responsible for the free radical scavenging effect.

The scavenging activity of different extracts of Andrographis paniculata on DPPH radicals is shown in table1. The IC_{50} value of petroleum ether, ethyl acetate, ethanol, hydroalcoholic extracts and the isolated andrographolide were found to be 8.7µg/ml, 7.4µg/ml, 6.5µg/ml, 5.3µg/ml and 5.2µg/ml respectively.

Table no. 1 DPPH scavenging activity of various fractions of *Andrographis paniculata* and andrographolide.

Various Fractions	%inhibition				
	5	10	15	20	25
	(Concentration in µg/ml)				
Petroleum ether	10 ± 1.0	15 ± 1.6	20 ± 2.2	25 ± 2.8	28 ± 3.2
Ethyl acetate	15 ± 1.5	20 ± 2.1	23 ± 2.5	29 ± 3.3	36 ± 4.0
Ethanol	25 ± 2.5	33 ± 3.4	40 ± 4.2	45 ± 4.8	58 ± 6.2
Hydroalcoholic	29 ± 2.9*	$36 \pm 3.7^{\#}$	$48 \pm 5.0^{\#}$	$52 \pm 5.5^{\$}$	$59 \pm 6.3^{\$}$
Andrographolide	31 ± 3.1*	$40 \pm .4.1^{\#}$	$50 \pm 5.2^{\#}$	$59 \pm 6.2^{\$}$	$65 \pm 6.9^{\$}$
Ascorbic acid	33 ± 3.3*	$45 \pm 4.6^{\#}$	$69 \pm 7.1^{\#}$	$75 \pm 7.8^{\$}$	$77 \pm 8.1^{\$}$

Values are expressed as mean ± SD for six individual experiments. Statistically significant difference is expressed as $^{\$}p<0.001$, $^{\#}p<0.01$, $*p<0.05$.

DPPH is a stable free radical, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts [41]. When DPPH encounters a proton – donating substance such as an antioxidant, the radical would be scavenged and the absorbance reduced. Based on this principle, the antioxidant activity of a substance can be expressed as an ability to scavenge the DPPH radical. Among the various extracts tested, andrographolide and hydroalcholoic extract were found to exhibit greater DPPH radical scavenging activity. The IC_{50} values of andrographolide and hydroalcoholic extracts were found to be nearer to that of standard ascorbic acid 5.0µg/ml.

The superoxide scavenging activity of the extracts with their IC_{50} values are shown in table 2. IC_{50} values of petroleum ether, ethyl acetate, ethanol, hydroalcoholic and andrographolide were found to be 11µg/ml, 9.3µg/ml, 8.5µg/ml, 7µg/ml and 6µg/ml respectively.

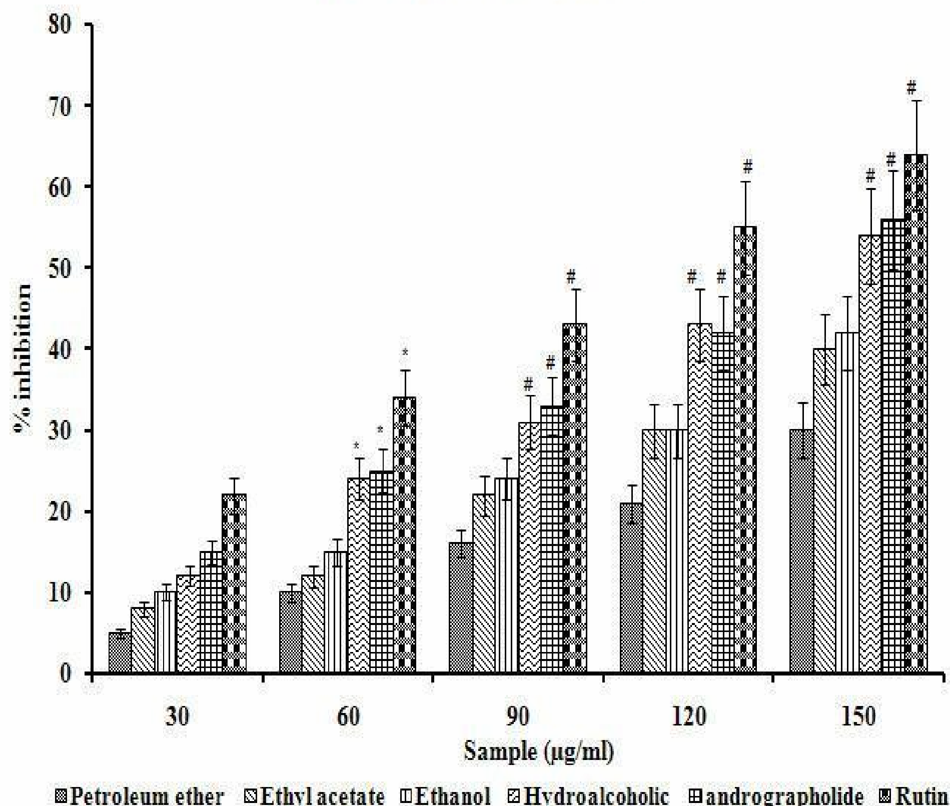
Table no. 2 Superoxide scavenging activity of various fractions of *Andrographis paniculata* and andrographolide.

Various Fractions	%inhibition				
	5	10	15	20	25
	(Concentration in µg/ml)				
Petroleum ether	12 ± 1.2	19 ± 2.0	22 ± 2.4	25 ± 2.8	40 ± 4.4
Ethyl acetate	15 ±1.5	21 ± 2.2	26 ± 2.8	32 ± 3.5	42 ± 4.6
Ethanol	19 ± 1.9	28 ± 2.9	36 ± 3.8	45 ± 4.8	52 ± 5.6
Hydroalcoholic	21 ± 2.1*	36 ± 3.7*	41 ± 4.3$^{\#}$	52 ± 5.5$^{\$}$	56 ± 6.0$^{\$}$
Andrographolide	29 ± 2.9*	38 ± 3.9*	43 ± 4.5$^{\#}$	60 ± 6.3$^{\$}$	64 ± 6.8$^{\$}$
Curcumin	32 ± 3.2*	40 ± 4.1*	45 ± 4.7$^{\#}$	68 ± 7.1$^{\$}$	70 ± 7.4$^{\$}$

Values are expressed as mean ± SD for six individual experiments. Statistically significant difference is expressed as $^{\$}p<0.001$, $^{\#}p<0.01$, $*p<0.05$.

Superoxide anion is a free radical created during energy metabolism in the human body. Superoxide anion is toxic to cells and tissues and can act as precursors to other reactive oxygen species [42]. It was found that all the extracts exhibited superoxide scavenging activity but more significantly hydroalcoholic extract and andrographolide. The IC_{50} value of andrographolide (6µg/ml) was found to be comparable to that of standard curcumin (5.7µg/ml).

Nitric oxide is a free radical that exhibits neumerous physiological properties and it is also implicated in several pathological states [43]. The nitric oxide scavenging activity of various solvent extracts is shown in fig.1. The NO scavenging activity is seen in the order of petroleum ether, ethyl acetate, ethanol, hydroalcoholic and of andrographolide with the IC_{50} values of 85µg/ml, 80µg/ml, 74µg/ml, 70µg/ml and 66µg/ml respectively.

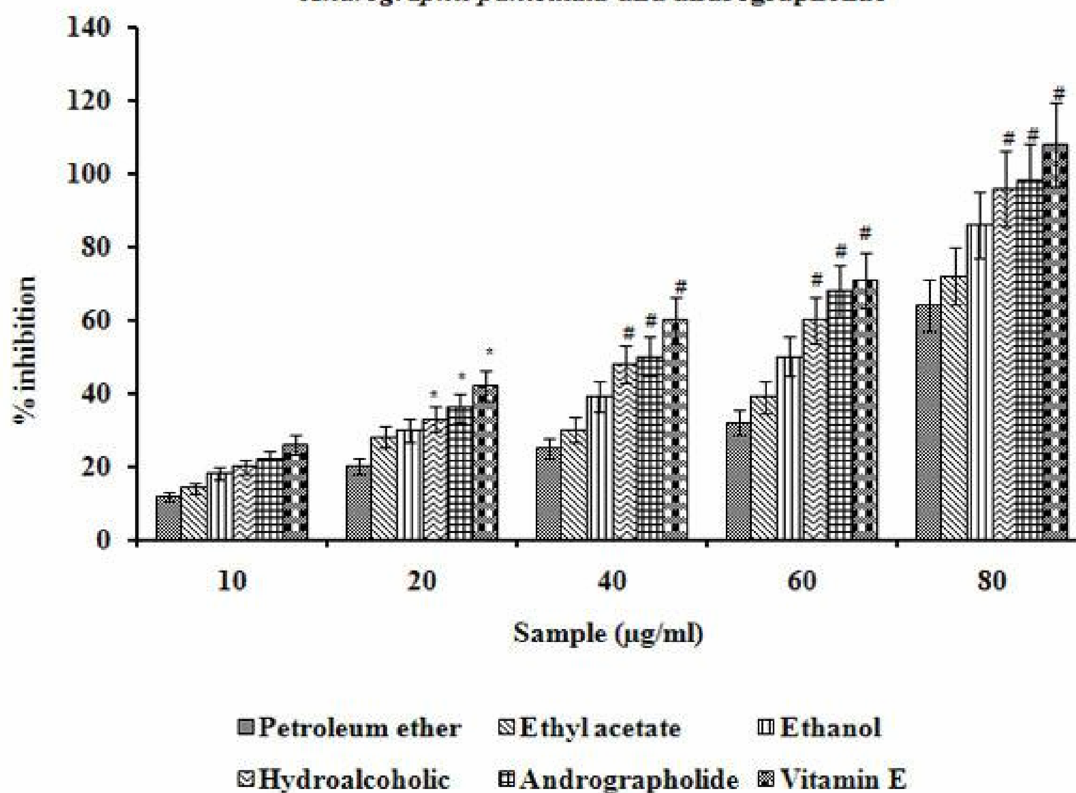


Values are expressed as mean ± SD for six individual experiments. Statistically significant difference is expressed as #p<0.01, *p<0.05 when compared to minimal dose.

Excess production of nitric oxide is associated with several diseases. NO is produced in various cells including neurons, endothelial cells and neutrophils by three isoforms of NO synthase, from L-arginine [44]. The nitric oxide scavenging activity of the various extracts shows that the isolated compound andrographolide exhibited maximum nitric oxide scavenging activity. When comparing the IC_{50} value of rutin 60µg/ml, andrographolide showed the highest nitric oxide scavenging activity when compared to the other solvent extracts.

The hydroxyl radical is extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology capable of damaging biomolecules of the living cells [45, 46]. Fig. 2 show the hydroxyl radical scavenging activity of various solvent extracts. The isolated andrographolide showed maximum scavenging activity with the IC_{50} value of 38µg/ml. The IC_{50} values of petroleum ether, ethyl acetate, ethanol and hydroalcoholic extracts were found to be 60µg/ml, 54µg/ml, 47µg/ml and 40µg/ml respectively. The IC_{50} value of vitamin E was found to be 34µg/ml.

Fig 2. Hydroxyl radical scavenging activity of various fractions of *Andrographis paniculata* and andrographolide



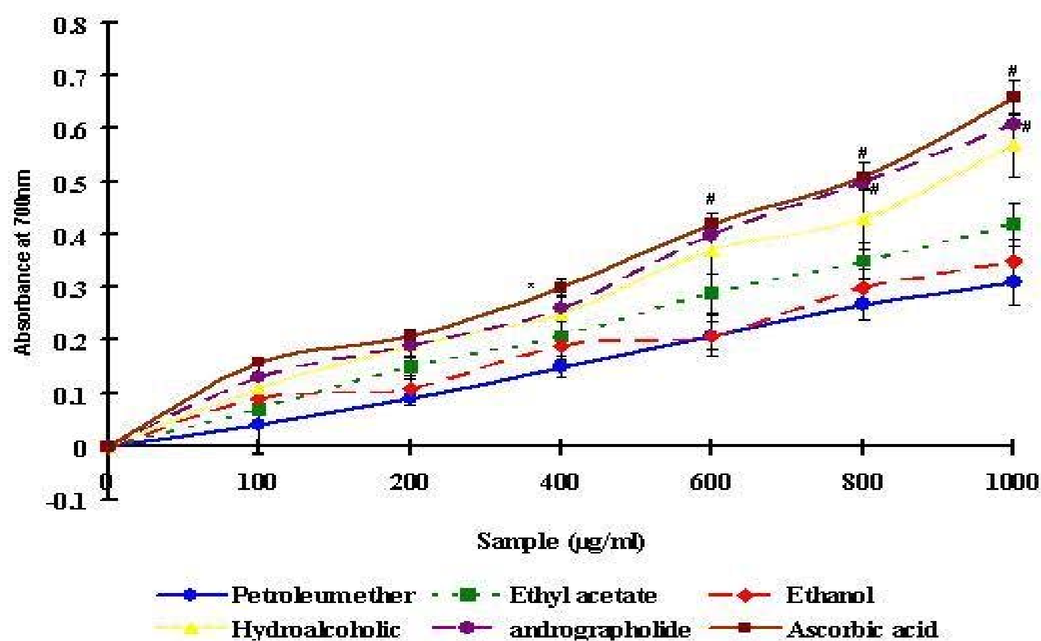
Values are expressed as mean ± SD for six individual experiments. Statistically significant difference is expressed as #p<0.01, *p<0.05 when compared to minimal dose.

Some compounds are capable of redox cycling the metal ions required for hydroxyl ions generation, thus increasing the radical production, exhibiting a pro-oxidant activity [47]. All the extracts have shown inhibitory activity against hydroxyl radical and there was a significant difference in their activities at similar concentrations. Among all the test compounds, andrographolide exhibited greatest scavenging activity. The IC_{50} value of andrographolide was comparable with that of Vitamin E.

Antioxidant activity of medicinal plant products has been reported to be attributed to their reducing power. The reducing power of the various extracts is shown in fig. 3 and the effect was more pronounced in andrographolide. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

The reducing ability of a compound generally depends on the presence of reductants [48], which exhibit antioxidative property by donating a hydrogen atom and breaking the free radical chain events [49]. The reducing ability was present in all the fractions but significantly in hydroalcoholic extract and in andrographolide.

Fig 3. Reducing power exhibited by various fractions of *Andrographis paniculata* and andrographolide



Values are expressed as mean ± SD for six individual experiments. Statistically significant difference is expressed as #p<0.01, *p<0.05 when compared to minimal dose.

H^+K^+ ATPase is a prime enzyme that influences secretion of acid in the stomach to aid digestion of proteins. We have found that andrographolide isolated from *Andrographis paniculata* exhibits maximum H^+K^+ ATPase inhibitory action and comparable to that of standard antiulcer drug omeprazole at similar concentrations.

Table 3 shows the H^+K^+ ATPase activity of sheep gastric parietal cells treated with different solvent extracts of *Andrographis paniculata* and omeprazole. Andrographolide exhibited maximum inhibition with the IC_{50} value of 27µg/ml when compared to that of other extracts. The IC_{50} value of petroleum ether, ethyl acetate, ethanol and hydroalcoholic extracts, andrographolide and omeprazole were found to be 18µg/ml, 23µg/ml, 25µg/ml, 26µg/ml, 27µg/ml and 29.5µg/ml respectively.

Table no. 3 H^+K^+ ATPase inhibitory activity of various fractions of *Andrographis paniculata* and andrographolide.

Various Fractions	%inhibition							
	10	20	30	40	50	60	70	80
	(Concentration in µg/ml)							
Petroleum ether	10.6 ± 1.06	19.5 ± 1.96	23.6 ± 2.37	26.8 ± 2.71	32 ± 3.6	33 ± 3.9	34 ± 4.0	35.2 ± 4.2
Ethyl acetate	14 ± 1.4	23.6 ± 2.37	25.2 ± 2.53	27 ± 3.0	33.6 ± 3.36	34 ± 3.5	38 ± 3.9	38.9 ± 4.1
Ethanol	16.8 ± 1.68	24.9 ± 2.5	26.4 ± 2.7	27.6 ± 2.8	34.9 ± 3.5	35 ± 3.6	40 ± 4.0	42.1 ± 4.3
Hydroalcoholic	17.7 ± 1.7*	25.3 ± 2.6*	28.7 ± 2.9*	29.6 ± 3.0*	35.4 ± 3.6*	36 ± 3.8*	45.4 ± 4.6*	46.4 ± 4.6*
Andrographolide	19.4 ± 1.94$^{\#}$	26 ± 2.7$^{\#}$	29.4 ± 3.0$^{\#}$	32.4 ± 3.4$^{\#}$	36.9 ± 3.9$^{\#}$	40.2 ± 4.2$^{\#}$	46.3 ± 4.7$^{\#}$	47.2 ± 4.8$^{\#}$
Omeprazole	20 ± 2.0$^{\$}$	26.2 ± 2.7$^{\$}$	30.13 ± 3.2$^{\$}$	34.7 ± 3.5$^{\$}$	37.5 ± 3.8$^{\$}$	45.4 ± 4.6$^{\$}$	48 ± 4.9$^{\$}$	55 ± 5.6$^{\$}$

Values are expressed as mean ± SD for six individual experiments. Statistically significant difference is expressed as $^{\$}p<0.001$, $^{\#}p<0.01$, $^{*}p<0.05$.

H^+K^+ ATPase is the proton pump responsible for gastric acid secretion [50, 51], and the final common pathway mediating secretion of hydrochloric acid by gastric parietal cells [52]. The enzyme typically located in the parietal cells, mediates the electroneutral exchange of intracellular H^+ and extracellular K^+ to achieve acid secretion when parietal cells are under the stimulation of secretagogues [53]. Gastric H^+K^+ ATPase is a membrane bound enzyme located in the apical membrane of parietal cells which pump protons into the gastric lumen using energy derived from the hydrolysis of ATP [50]. Inhibition of this enzyme results in the inhibition of acid secretion. Thus, H^+K^+ ATPase inhibition test constitutes the best pharmacological target to screen new drugs for ulcer.

In this investigation, it was found that the activity of H^+K^+ ATPase was inhibited *invitro* by the andrographolide in a significant manner when compared to the other extracts. This is on par with omeprazole indicating the potential effect of test material as an inhibitor of acid secretion in stomach parietal cells which can be accounted for its antiulcer activity. The hydroalcoholic extract also was found to inhibit the enzyme significantly when compared to other extracts.

Antioxidants are vital substances which posses the ability to protect the body from free radical induced oxidative stress. Polyphenols present in certain medicinal and dietary plants have helped in preventing oxidative damage thus increasing interest towards natural antioxidants [54]. Phytochemicals are required from a spectrum of sources for their antioxidant role to protect tissues from activities that manifest themselves into chronic diseases [55].

The quantitative determination of phytochemicals in the hydroalcoholic extract of *Andrographis paniculata* was found to contain flavonoids (total), alkaloids, xanthones, tannins and triterpenes at the level of 0.0791%, 0.0989%, 0.1621% and 2.156%. triterpenes was present abundantly (2.156) in *Andrographis paniculata* as shown in table 4.

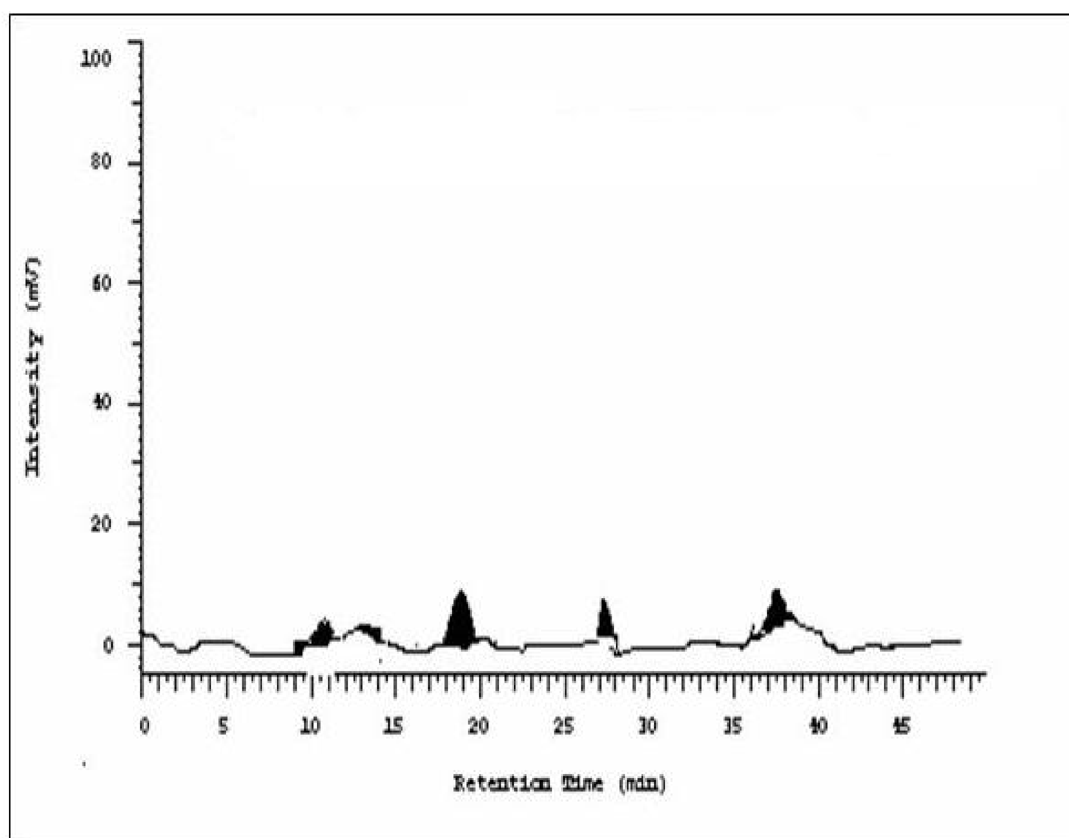
Flavonoids that contain multiple $OH^·$ stimulation have very strong antioxidant activities against peroxyl radicals [56]. Recent reports and extensive literature survey indicate that many flavanoids and phenolic anti-oxidants possess anti-ulcerogenic and wound healing activity [57]. Some of the flavanoids have been shown to increase the mucosal content and prostaglandin synthesis [58]. Diterpenoids and flavonoids are the main chemical constituents of *Andrographis paniculata* and these compounds are believed to be responsible for the medicinal activities of the plant [59, 60].

Table no.4 Quantitative determination of phytochemicals in the hydroalcoholic extract of *Andrographis paniculata*

DESCRIPTION	A BROWN COLOURED LIQUID
IDENTIFICATION	POSITIVE FOR FLAVONOIDS, ALKALOIDS, XANTHONES, TANNINS AND TRITERPENES
FLAVONOIDS (TOTAL)	0.0791% W/W BIOCHIN A - 0.0178% QUERCETIN - 0.056% FORMONONETIN - 0.011%
ALKALOIDS	0.0989% W/W
XANTHONES	0.0856% W/W
TANNINS	0.1627% W/W
TRITERPENES	2.156% W/W

A typical HPLC-UV chromatogram of hydroalcoholic extract of *Andrographis paniculata* was shown in fig.4. Four components were observed in the chromatogram as peaks. Two were identified as Quercetin (18.08 R.T) and Formononetin (39.44) and their retention time being similar with those of authentic standards.

Fig.4 Detection of flavonoids by HPLC-UV chromatogram



Increased gastric acidity and inflammation are considered to be important contributing factors in the pathogenesis of gastric ulcers [61]. Table 5 presents the ulcer score measured in the gastric wall of the experimental animals. The ulcer score was significantly reduced in rats pretreated with 3mg/ kg b wt of andrographolide when compared with other solvent extracts. The ulcer reducing effect was comparable with that of the standard drug ranitidine. Ulcer score was reduced significantly in all the experimental model of ulcer.

Table no.5 Ulcer score in ulcerogen animals pretreated with andrographolide and various solvent extracts of *Andrographis paniculata*.

Treatment protocol	**Ulcerogens (Ulcer score)**		
	Ethanol	**Aspirin**	**Pylorus ligation**
Rats treated with ulcerogens	4.63 ± 0.46	4.01 ± 0.44	4.70 ± 0.52
Rats pretreated with 200mg/kg b wt of petroleum ether extract of *Andrographis paniculata* for 30 days +Ulcerogen	2.6 ± 0.27* (44%)	3.1 ± 0.34* (23%)	2.5 ± 0.27* (47%)
Rats pretreated with 200mg/kg b wt of ethyl acetate extract of *Andrographis paniculata* for 30 days +Ulcerogen	2.7 ± 0.31* (42%)	2.9 ± 0.34* (28%)	3.0 ± 0.36* (36%)
Rats pretreated with 200mg/kg b wt of ethanol extract of *Andrographis paniculata* for 30 days +Ulcerogen	1.8 ± 0.18* (61%)	2.0 ± 0.22* (50%)	1.9 ± 0.21* (60%)
Rats pretreated with 200mg/kg b wt of hydroalcoholic extract of *Andrographis paniculata* for 30 days +Ulcerogen	1.0 ± 0.10* (78%)	1.2 ± 0.13* (70%)	1.1 ± 0.12*(77%)
Rats pretreated with 3mg/kg b wt of andrographolide for 30 days +Ulcerogen	0.8 ± 0.08* (83%)	0.7 ± 0.07* (83%)	0.9 ± 0.09*(81%)
Rats pretreated with 30mg/kg b wt of omeprazole for 30 days +Ulcerogen	1.0 ± 0.11* (78%)	1.10 ± 0.12* (73%)	1.15 ± 0.13*(76%)

Values are expressed as mean ± SD for six animals in each group. Statistically significant difference is expressed as *p<0.001. (Percentage in parenthesis) Groups are compared as: Ulcerogens vs Rats pretreated with 200mg/kg b wt of petroleum ether extract, ethyl acetate extract, hydroalcoholic extract of *Andrographis paniculata*, 3mg/kg b wt of andrographolide & 30mg/kg b wt of omeprazole for 30 days +Ulcerogen.

Studies suggest that the ethanol induced damage to the GI mucosa starts with microvascular injury, namely a disruption of the vascular endothelium resulting in increased vascular permeability,edema formation and epithelial lifting [62]. Administration of aspirin produces severe gastric haemorrhagic erosions and has not been found to increase the aggressive factors (acid and pepsin) but significantly decreased the gastric output because of so called back diffusion of hydrochloric acid through the broken barrier inhibition of mucosal blood flow and acute inflammation [63, 64]. In pylorus ligated rats gastric acid is associated with severe ulceration of the rat gastric mucosa [65]. Pepsin and hydrochloric acid (HCl) are important for the formation of pylorus ligated ulcers [66]. The rats treated with various solvent extracts / 3mg/kg b wt of andrographolide shows significant reduction in the ulcer score.

Invivo study revealed that andrographolide and the hydroalcoholic extract of *Andrographis paniculata* acted as potent ulcer reducing agents and the effect was comparable to that of standard drug omeprazole. The ulcer score was reduced significantly in all the experimental ulcer models studied. However the detailed study on the level of prostaglandins and gastric mucin in drug treated animals is essential to confirm the antiulcer property.

Conclusion

It can be concluded that that the hydroalcoholic fraction of the aerial parts of Andrographis paniculata and andrographolide isolated from the plant possess potent H^+K^+ ATPase inhibitory activity invivo as well as invitro. The potent free radical scavenging activity of the test drugs may probably influence the antiulcer property by preventing the formation and the harmful action of toxic oxygen free radicals on gastric mucosa. The H^+K^+ ATPase inhibitory activity may also be accounted for gastroprotective activity.

Acknowledgment

The authors thank Dr. Jayaraman for having identified and authenticated the plant.

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