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Original Research Article

Anti - ACE Activity of Seasonal Fruits in Fluoride Toxicity: An In Vitro Investigation

Narasimhacharya, A.V.R.L., Krutika L. Bhole, Himanshi Bhatt

Laboratory for Animal sciences, Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Gujarat, India.

Corresponding Author: A.V.R.L. Narasimhacharya

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ABSTRACT

Fluorosis, caused by a chronic exposure to fluoride is a worldwide health problem known to affect various organ systems. The present study was aimed at investigating the effect(s) of extracts of seasonal fruits (Indian goose berry, star fruit, guava and wood apple) on angiotensin- converting enzyme (ACE) activity in the lung extracts exposed to different concentrations of fluoride. The lung extracts showed an increasing ACE activity with increasing concentrations of fluoride (2.5-10ppm) with highest ACE activity at 10ppm of fluoride. A reduction in the ACE activity was observed when the fluoride exposed (10ppm) lung extracts were incubated with fruit extracts; wood apple extract exerted a maximum ACE inhibitory activity (28.9%) followed by star fruit, guava and goose berry (20.1 %,19% and 15.5% respectively). A phytochemical analysis and the antioxidant potential of the fruits indicated that the fruits have a high antioxidant potential that correlated well with phytochemical content (phenols, flavonoids, saponins and ascorbic acid) of the fruits. It is suggested that the consumption of these seasonal fruits could be useful in regulating the ACE activity in fluoride endemic areas.

Keywords: ACE; fluoride; hypertension; seasonal fruits; antioxidants

INTRODUCTION

Hypertension has become a major cause of morbidity and mortality worldwide and it is now ranked third as a cause of disability-adjusted life years (Ezzati et al. 2002). In India, 20-35% of the population has been reported for hypertension (Pradeepa & Mohan 2008). One major control mechanism for blood pressure is the renin-angiotensin-aldosterone system or RAAS (Ondetti & Cushman 1982), in which angiotensin converting enzyme (ACE) is a vital player. ACE (peptidyldipeptide hydrolase EC 3.4.15.1) is an exopeptidase that catalyses the hydrolytic removal of carboxy-terminal dipeptide residues from polypeptide substrates (Erdos 1975). ACE plays a crucial role in regulation of blood pressure as it promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II and inactivates bradykinin, a vasodilator that depresses the renin-angiotensin system. ACE is also involved in the release of a Na-retaining steroid, aldosterone from the adrenal cortex, which has a tendency to increase blood pressure (Li et al. 2003). Therefore inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Various ACE inhibitors are available in market such as captopril, enalapril, lisinopril and ramipril etc. However, these pharmaceutical products are not without side effects such as cough, taste disturbances, skin rashes and allergic reactions.

A number of chemical compounds are known to affect the biological activities. Excessive intake of fluoride is associated with a wide range of adverse effects (Whitford et al. 1979; Dobaradaran 2008; Rahmani et al. 2010; Varol et al. 2010). Fluoride is known to occur at elevated concentrations in a number of parts of the world often leading to a significant adverse impact on public health and well-being. Moreover it has been mentioned that fluoride accumulates not only in bones, but also to a lesser extent, in soft tissues particularly in cardiovascular system (Scarpa et al. 1993; Xu et al. 1997). Higher risk of hypertension in those residing in areas with relatively higher water fluoride concentration has been reported (Liyan et al. 2013). Intake of natural sources and their ability to treat hypertension in fluoride endemic areas is unexplored. Earlier report on seasonal fruits in in-vitro studies using mammalian lung showed ACE inhibitory activity (Narasimhacharya et al. 2009). Consumption of a diet rich in fresh fruits and vegetables has been associated with a number of health benefits. This beneficial effect is believed to be due, at least partially, to the action of antioxidant compounds, which reduce oxidative damage in the body (Lana & Tijskens 2006). Multiple biological effects like antioxidant, anti-inflammatory, anticarcinogenic and free radical scavenging activities are reported for phytochemicals like flavonoid and phenolic components from the natural sources (Rice Evans & Miller 1996).

In the present study, in-vitro anti-ACE activities of four seasonal fruits (Indian Goose berry, Star fruit, Guava and Wood apple) on rabbit lung extracts exposed with fluoride were investigated. The fruits extracts were also tested for their phytochemicals and antioxidant properties.

MATERIALS AND METHODS

Fruit collection and extract preparation

Averrhoa carambola (Star fruit) and *Limonia acidissima* (Wood apple) were procured from local market while *Psidium guajava* (Guava) and *Emblica officinalis* (Indian goose berry, Amla) were purchased from Anand Agriculture University, Anand, India. All the fruits were washed with tap water, de-skinned and pulp was weighed, homogenized with 50 mM Borate buffer, pH 8.3, centrifuged at 10,000 g at 4° C for 20 minutes and the supernatant was collected. The extracts were stored at -20°C until further use.

Reagents and Chemicals

Rabbit lung acetone powder and Hippuryl-L-histidyl-L-leucine (HHL) were procured from Sigma Aldrich India. Cyanuric chloride was purchase from Alfa Aesar (Lancaster). All other reagents and chemicals used were of Analytical Grade.

Lung Extract preparation

One gram of lung powder was weighed and homogenized in 10 ml of 50 mM borate buffer at pH8.3 and centrifuged at 10,000 g for 50 min at 4^0 C. The supernatant was transferred to microcentrifuge tubes and stored at $-20^{0\ C.}$

ACE activity

ACE activity was measured using the method described by Schnaith et al. (1994). Hippuryl-L-histidyl-L-leucine (HHL) was used as substrate. The reaction mixture contained 0.2 ml of 5mM HHL prepared in incubation buffer, pH 8.3 and lung extract. Lung extract in a volume of 50 µl was added to initiate the reaction and was stopped by adding 1.5ml of 100mM HEPES, pH 9.0, containing 2.5mM EDTA, 0.75 ml of 136mM cyanuric chloride prepared in 1, 4-dioxan was added to the reaction mixture and mixed vigorously and centrifuged for 15 min at 3000 rpm. The absorbance of the yellow colour that developed was measured at 405nm.

One unit of ACE was defined as the amount of enzyme catalyzing the release of 1 micromole of hippuric acid from HHL per minute at 37º C. The ACE-inhibitory activity was calculated using the equation: Inhibition activity (%) = [1 - (A - B) / (C - D)] × 100; A is the absorbance of the solution with ACE substrate, and sample; B is the absorbance of the solution with ACE

and the sample but without substrate and C is the absorbance of the solution with ACE and substrate but without the sample. D is the absorbance of the solution containing only the substrate (Jang and Lee, 2011; Min-Gu Kang et al. 2013).

In- vitro effect of Sodium fluoride and fruit extracts on ACE activity

ACE activity was determined using different concentrations of sodium fluoride (2.5, 5, 7.5, 10 ppm). Equal amounts of ACE (Rabbit lung homogenate) and different concentrations of Sodium fluoride were incubated for 10 minutes at 37º C. After incubation the ACE activity was assessed as described above. Further, suitable aliquots of fruit extracts (125 mg/ml) were added to the incubation mixture to determine the ACE activity and compared with Captopril's (a standard anti-hypertensive drug) suppressive action on ACE.

Phytochemical analysis

A quantitative analysis of fruits was carried out for phenol, flavonoid, saponin, total ascorbic acid and protein content. Phenol content was determined using Folin-Ciocalteu reagent (Thimmaiah 1999). Total flavonoid content of fruits was determined using Aluminium chloride colorimetric method as described by Zhishen et al. (1999). The saponin content of the fruits was determined using Vanillin- sulphuric acid reagent as described by Ebrahimzadeh & Niknam (1998), using Schaffert and Kingsley (1955) method total ascorbic acid was measured and protein content was determined using Lowry et al. (1951).

Antioxidant analyses

Ferric reducing antioxidant power assay (FRAP) for fruits extract was performed as per the procedure described by Benzie & Strain (1996). DPPH and ABTS radical scavenging activity of fruits was measured using the method described by Brand et al. (1995) and Arnao et al. (2001) respectively. The hydroxyl radical scavenging ability of fruit extracts were estimated according to the method of Smirnoff & Cumbes (1989). Trolox was used as standard for all the antioxidant measurements.

Data analysis

Difference between the ACE inhibitory activity, phytochemical measurement and antioxidant capacity of the fruit extracts were tested by ANOVA , Tukey's test was used to determine significant difference ($p < 0.05$) by using SPSS for window (version 16.0).

RESULTS

Fluoride- exposure affects ACE activity in lung extract

The rabbit lung extracts were exposed to different doses of sodium fluoride (2.5, 5, 7.5 and 10 ppm) and the ACE activity was investigated. With the increase in the concentration of the sodium fluoride, an increased ACE activity was observed. Among the different concentrations of fluoride 10 ppm (F10) exhibited highest ACE activity. Thus 10 ppm of fluoride concentration exposed lung extract was selected for further study (Table 1).

Anti ACE activity of fruits homogenate in fluoride exposed rabbit lung extract

All the fruit extract homogenates (125 mg/ml) showed anti ACE activity in lung extract exposed with 10 ppm of fluoride toxicity. Among all the fruit extracts tested, wood apple pulp extract exhibited highest anti ACE activity (~29%) followed by star fruit (20%), guava (19%) and Indian goose berry (~16%) (Table 2).

Phytochemical analysis of fruit extracts

Indian goose berry showed highest flavonoid, saponin, and total ascorbic acid and protein contents as compared to other fruit extracts. The phenol content was observed to be highest in guava fruit extract as compared to other fruits (Table 3).

Antioxidant profiles of fruit extracts

Among the fruits, Amla fruit extract possessed high FRAP DDPH and ABTS radical scavenging activity. The hydroxyl radical scavenging activity was highest in wood apple extract compared to other fruit extracts (Table 4).

Table 1: Fluoride treatment on lung extract

Fluoride treatment groups	ACE enzyme activity	% Inhibition in ACE activity
Control (Rabbit lung extract)	2.3755^{a}±0.10923	-
F2.5	2.8480^{b}±0.11444	19.89
F5	2.9924^{b}±0.01137	25.96
F7.5	2.9464^{b}±0.07983	24.03
F10	3.1302^{b}±0.07956	31.77

Data presented are Mean ± SEM (n= 3) (F-2.5, 5, 7.5 and 10 ppm: Rabbit lung extract treated with 2.5, 5, 7.5 and 10 ppm of sodium fluoride.) Different alphabets (a,b) are statistically significant at $p < 0.05$

Table 2: Effect of Fruit extracts on Fluoride treated lung extract

Fruit extracts treated with 10 PPM Sodium fluoride	ACE Enzyme activity	% Inhibition in ACE activity
Control	2.3755^{bc}±0.10923	-
F10	3.1302^{d}±0.07956	31.77
Indian goose berry (125 mg/ml)	2.6446^{c}±0.05372	15.51
Starf ruit(125 mg/ml)	2.5002^{bc}±0.11810	20.12
Wood apple(125 mg/ml)	2.2246^{b}±0.04101	28.93
Guava(125 mg/ml)	2.5330^{bc}±0.10561	19.07
Captopril(10 µM)	0.6234^{a}±0.02859	80.08

Data presented are Mean ± SEM (n= 3) (F 10: rabbit lung extract treated with 10 ppm sodium fluoride) Different alphabets (a,b,c,d) are statistically significant at $p < 0.05$

Table 3: Phytochemical profiles of fruit extracts

Fruits	Phenol*	Flavonoid*	Saponin*	Total ascorbic acid*	Protein*
Indian goose berry	0.653^{c} ± 0.001	24.39^{c} ± 0.208	22.31^{c}±0.039	1.00^{d} ± 0.004	234.78^{c} ± 3.444
Star fruit	0.082^{a} ± 0.001	1.64^{a} ± 0.137	20.31^{b} ± 0.009	0.26^{a}± 0.003	7.68^{a} ± 0.156
Guava	0.665^{d} ± 0.000	4.89^{b} ± 0.092	20.37^{b} ± 0.019	0.90^{c}± 0.003	22.40^{b}± 0.132
Woodapple	0.542^{b} ± 0.003	4.70^{b} ± 0.074	13.14^{a} ± 0.009	0.50^{b} ± 0.020	28.51^{b}± 0.843

Data presented are Mean ± SEM, *= mg/g of FW (FW= fresh weight), Different alphabets (a, b, c and d) in column are significantly different from each other at $p< 0.05$.

Table 4: Antioxidant potentials of fruit extracts

Fruit	FRAP@	DPPH**	ABTS**	Hydroxyl radical scavenging activity**
Indian goose berry	1.870^{c} ± 0.000	96.506^{c}± 0.107	99.966^{c} ± 0.016	33.146^{a} ± 4.528
Star fruit	0.589^{a} ± 0.006	4.495^{a} ± 0.767	27.480^{a} ± 0.605	77.070^{c} ± 4.674
Guava	1.837^{c}± 0.000	94.566^{c}± 0.241	99.156^{c} ± 0.035	58.210^{b} ± 1.936
Wood apple	1.579^{b} ± 0.015	28.758^{b}± 0.615	45.463^{b} ± 2.980	89.820^{d} ± 1.740

Data presented are Mean ± SEM, @ = mg/g of FW, ** = % inhibition, Different alphabets (a, b, c and d) within the column are statistically significantly at $p <0.05$.

DISCUSSION

Consumption of fluoride through ground water is known to have deleterious effects on health systems and affects major metabolic pathways. An ecological study in Iranian population showed a positive relationship between fluoride concentrations in ground water and blood pressure (Amini et al. 2011). In present study there was increase in ACE activity of rabbit lung extracts in a dose dependent manner on exposure to sodium fluoride i.e. (2.5 ppm-10 ppm). The highest ACE activity i.e. 3.1302 (+31.77% increase compared to control rabbit lung extract was found in 10 ppm sodium fluoride exposed lung extract) (Table 1). The interaction between fluoride and increased ACE activity indicated the possible incidences of hypertension at local level. An earlier study by Thaweboon et al. (2003) on human dental pulp cells in in-vitro system demonstrated increased alkaline phosphatase activity at lower doses of fluoride, while higher concentration of fluoride had negative effect on the dental pulp cells. Thus the effect of fluoride and its interaction with alkaline phosphatase activity is neither coherent nor transparent (Adamek et al. 2005). When F10 exposed lung extracts were tested with different fruit extracts and their ACE activity revealed that all the fruits exhibited anti- ACE activity at varying degrees at fixed dosage of 125mg/ml. The pulp extract of wood apple showed a decrease in ACE activity by 29%, while star fruit, guava and Indian goose berry reduced ACE activity of F10 by 20%, 19 % and ~16 % respectively. No statistical difference was observed in the star fruit and guava fruit extracts' anti- ACE activity. Extract from Indian goose berry showed anti-ACE activity which was statistically similar to star fruit and guava but

statistically different from wood apple extract.

These observations clearly indicate the potential of the fruits to reduce the ACE activity (although at a greatly lower level compared to Captopril's anti-ACE activity) and are suggestive of benefits of consumption of these fruits in fluoride endemic areas.

The phytochemical analysis indicated that goose berry is rich in phytochemical contents (flavonoids, saponins, total ascorbic acid and proteins) compared to other fruits. A number of plant compounds such as terpenoids, tannins, fatty acids, oligosaccharides, peptides/ amino acids, alkaloids and flavonoids etc are known to act as ACE inhibitors (Nyman et al. 1998; Lacaille-dubois et al. 2001; Braga et al. 2007). One of the studies on pomegranate fruit juice showed inhibitory effect of fruit juice on oxidative stress and serum ACE activity, which was attributed to the tannin content in juice (Aviram 2002). Studies conducted on different types of wines showed peptide and phenolic components act as ACE inhibitors in in-vitro system (Pozo-Bayón et al. 2005). Additionally, an isolated peptide Phe-Val-Asn-Pro-Gln-Ala-Gly-Ser from sunflower protein also exhibited ACE inhibitory activity (Megías et al. 2004). Guava fruit extract showed highest phenol content as compared to other fruit extracts. On the other hand, flavonoids, saponins, total ascorbic acid and protein content was found to be highest in Indian goose berry extract (Table 3). Wood apple extract exhibited higher anti - ACE activity while the phytochemical components were at moderate level. These observations indicate the involvement of other compounds/ factors. For instance, Know et al., (2008) claimed that unknown factors may be responsible for ACE inhibitory activity since phenolic content of the eggplant did not correlate with ACE inhibitory activity in in-vitro conditions. Besides, fruits are known to be excellent source of minerals and vitamins (Nahar et al. 1990). Minerals such as K^+, Ca^{2+}, Na^{2+} etc are required in human diet for normal healthy life (San, 2009). Epidemiological studies also suggest intake of K^+ and blood pressure are inversely related (Barri & Wingo 1997) and several studies associated blood pressure with intake of Mg^{2+}, K^+, Ca^{2+}, fiber and protein (Harlam et al. 1984; Ascherio et al. 1992; Stamler et al. 1996; Stamler et al. 1996). The present investigation does not permit a direct correlation between the phytochemical content and the anti-ACE activities of the fruit extracts although all fruits exhibit the anti-ACE (ACE inhibitor) activity.

Oxidative stress has been implicated in chronic diseases including hypertension (Montezano & Touyz 2012). Although sole cause of hypertension is not only oxidative stress but also elevation of blood pressure due to additive effect of oxidative stress in presence of pro-hypertensive factors. Consequently oxidative stress is familiar in its course of action on fluoride exposure in in-vitro studies on different types of cells, different organ systems in animals and in human populations living in fluoride endemic areas (Barbier et al. 2010). It is widely accepted that fruits and vegetables have many beneficial properties (Hertog et al. 1995). Their positive influence is attributed to some natural antioxidant phyto-nutrients (Rice-Evans & Diplock 1993; Halliwell 1994; Rice- Evan & Miller 1994; Rice- Evan et al. 1996). The Indian goose berry extract showed highest antioxidant capacity compared to other fruit extracts. On the other hand hydroxyl radical scavenging activity of wood apple extract was highest compared to other fruit samples. The antioxidant activity of fruit extracts correlated with the phytochemical composition of the fruits respectively. Therefore is could be seen from the present study that all the four fruits possess antioxidant activity associated with phytochemical content. In conclusion, the findings of the present study revealed that sodium fluoride elevates the ACE activity in the lung extracts. All the four fruits tested

showed reduction in the ACE activity in presence of sodium fluoride. Moreover all the fruits possess phytochemicals which could possibly enhance their antioxidant potential that could reduce the oxidative stress. Therefore, it can be concluded that dietary intake of tested fruits could be useful in fluoride endemic areas with incidences of hypertension.

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