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Full Length Research Paper

Evaluation of *in vitro* antioxidant, reducing, lipoxygenase and ACE inhibition activity of polyherbal drug linkus

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Oxidative, reductive, lipoxygenase and angiotensin converting enzyme (ACE) activities are the condition where there is an inequity among concentrations can cause a multiple pathological effects. Vast results of medical plants and the remarkable contribution on humans has been observed since centuries. The current study has showed the antioxidant, lipoxygenase, ACE inhibition, urease activity and reducing ability *in vitro* on linkus formulation, including lozenges and syrup. The anti-oxidant activities was assessed by scavenging ability of the linkus on free radical (DPPH; $C_{18}H_{12}N_5O_6$) 2,2'-diphenyl-1-picryl hydrazyl. For lipoxygenase measurements, purified lipoxygenase with lionoleic acid as substrate was used for the activity, however for lipoxygenase activity the thiocholine and diothiobisnitrobenzoic acid (DTNB) was used. Indophenol method has been used for determination of urease activity, however the reducing ability has been assessed by the conversion of ferric into ferrous state. *In vitro* results of linkus syrup as compared to standard showed good anti-oxidant and reducing ability. Moderate activity of urease, lipoxygenase and ACE inhibition were observed with comparison of standard. These activities of polyherbal formulation might be helpful for reducing cough and related symptoms.

Key words: Antioxidant activity, reducing ability, urease activity, lipoxygenase and ACE inhibition, linkus.

INTRODUCTION

Reactive oxygen species (ROS) is the byproduct of oxidative stress under the physiological conditions. These extreme ROS accretion will lead to cell damage, such as damage to proteins, DNA, and lipid membranes. The cell injury/damage is initiated by ROS and has been associated with the development of numerous disease

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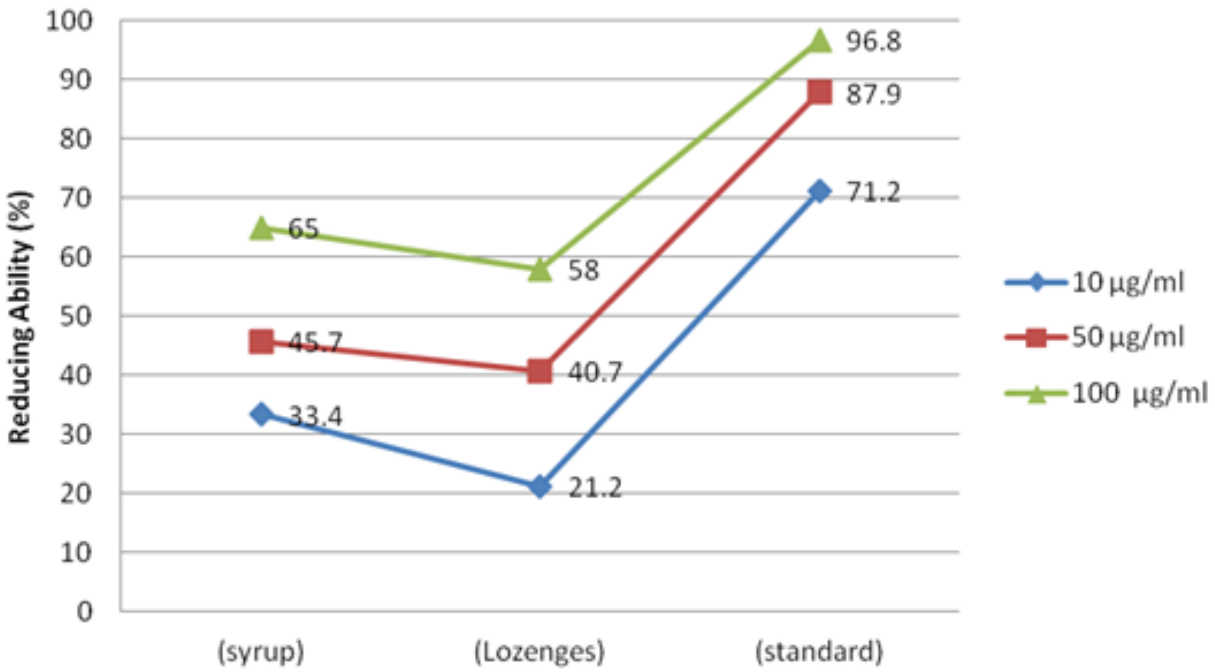

Figure 1. *In vitro* antioxidant activity of LInkus formulation including lozenges and syrup w.r.t standard.

injury/damage is initiated by ROS and has been associated with the development of numerous disease conditions, such as diabetes, atherosclerosis, cancer and cardiovascular disease etc. Its comprising multiple free radicals and exogenous factors play vital role (Finkel et al., 2000).

ROS *in vivo* inside the cell membrane performs multiple mechanism by sunlight or by different chemical and metabolic process, including DNA damage, carcinogenesis etc (Gyamfi et al., 1999; Ganapathy et al., 2011; Gutteridge and Halliwell, 2000; Halliwell, 2001). The free radical, chemicals and toxins creates effects in immune system and are declared as the major contributor of free radicals in the oxidation process (Halliwell, 1994; Kuhnan, 1976; Kumpulainen and Salonen, 1999; Younes, 1981).

Urease is the prominent agent for gastrointestinal track (GI) and help to inhibit *Helicobacter pylori*. Urease also acts directly as virulence factor in infections other than GI, including urinary tract both in humans and animals (Ghous et al., 2010; Halliwell et al., 2008; Nabati et al., 2012). Lipoxygenase is the significant main enzyme for biosynthesis of leukotriene and different potential drug to cope with asthma, arthritis, circulatory diseases etc (Wasserman et al., 1991) (incorporated inside text). Consequently, over a decade, the foremost effort has invested and many vitro active like 5-lipoxygenase inhibitors has developed (Ford-Hutchinson, 1991; Batt, 1992; McMillan and Walker, 1992; Ford-Hutchinson et al., 1994). ACE inhibitors are known to induce dry cough. There has an observation that prostaglandins with others are responsible for this effects but the statement is still controversial (Morice et al., 1987; Gilchrist et al., 1989; Fox et al., 1996).

Naturally occurring antioxidants have effective pharmacological action, including less toxicity and price effectiveness. Multiple plant products, including terpines and phenols also have this activity too (De Souza et al., 2007; Lin and Yin, 2007; Rice-Evans et al., 1996). This present study was based on linkus syrup and lozenges which have a wonder blend of polyherbs, including *Glycyrrhiza glabra, Adhatoda vasica, Viola odorata, Piper longum, Hyssopus officinalis* and *Alpinia galangal* (Appendix 1 and 2). The study focused on anti-oxidant and reducing ability on linkus dosage forms and proved the strength of activity inside the herbal formulation (Figure 1). By proving the activities, it claims that linkus might work on associated symptoms of cough and respiratory tract.

METHODOLOGY

Plant material and techniques

Herb extracts and mentioned chemical constituents were the part of Linkus lozenges and Syrup (Figure 2). Major specification included organoleptic evaluation, qualitative reaction of glycyrrhizic acid, tanning agents and ascorbic acid. Quantitative determination with spectrophotometric evaluation was observed. The total flavonoids contents as luteolin-7-glucoside were not less than 0.080 mg/lozenge (Zeeshan et al., 2014).

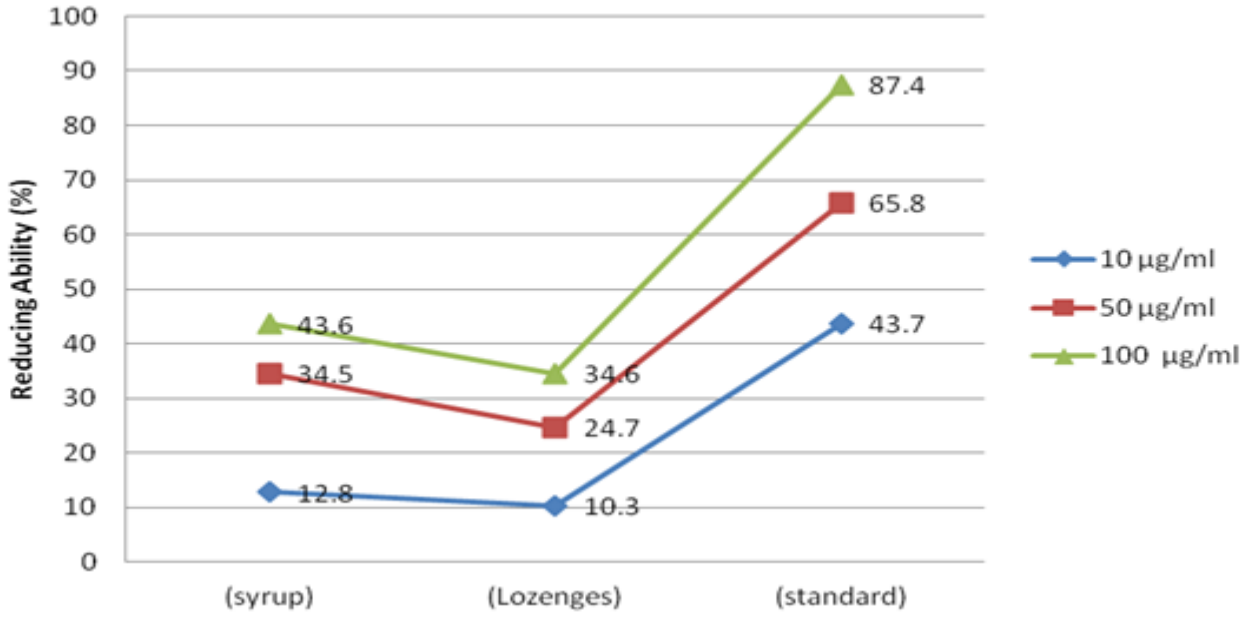

Figure 2. Linkus lozenges and syrup reducing ability w.r.t standard.

Preparation of plant extract

Individual herbs were taken separately, cleaned, grind, weighed and distill water added together with sugar and liquid glucose. Ingredients were transferred from weighing tank to storing tank with temperature range from 110 to 120°C for syrup and 60 to 80°C for lozenges. For lozenges, boiling and vacuum cooking was needed. Kneading, roping and sizing were the next step with the help of uni-plast machine. Lozenges were finally passed through cooling tunnel to obtain the desired hardness. For syrup, hot water filtrate was evaporated via a condenser. Methyl paraben, and Propyl paraben together with flavoring agents were added after completion (Zeeshan et al., 2014)

Chemicals and Reagents

All chemicals were high performance liquid chromatography (HPLC) grade. For reduction, 1, 1-diphenyl-2-picrylhydrazyl was used and obtained from Merck, Pakistan, 2,2'-diphenyl-1-picryl hydrazyl (DPPH) was obtained from Sigma-Aldrich Chemie (Buchs, Switzerland) and used for anti-oxidant activity.

Scavenging activity by DPPH radical

The antioxidant activity was measured by the scavenging aptitude of the syrup and capsules on free radical (DPPH). Antiradical activity analyzed depended on the reduction of DPPH. Its free radicals showed strong absorption at 517 nm due to odd electrons. When this electron was paired in the company of hydrogen donor, for example any antioxidant, the absorption strength decreased and color changed from purple to yellow, with respect to the number of electrons captured (Gülçin et al., 2005). For performing the process, 2, 2-Diphenyl-1-(2, 4, 6-trinitrophenyl) hydrazyl (M.W = 394.24) (Sigma) was prepared in ethanol in a concentration of 3 mM. Each well in 96-well plate was labelled as control, blank and test compound of various concentrations. DPPH solution (95 µl) was added in the labeled wells. The test compound (5 µl) of concentration 10 to 1000 µM in dimethyl sulphoxide (DMSO) was then added in DPPH solution and reaction mixture was mixed for few seconds. The reaction took place in wells when 96 well plates were incubated at 37°C for 30 min. The micro titre plate was read at the absorbance of 515 nm (Spectramax plus 384 Molecular Device, USA) after 30 min. The percentage of radical scavenging activity was considered with respect to DMSO treated control. Butylated hydroxyanisole (BHA) was taken as standard. The DPPH activity was performed with the help of the following equation:

DPPH radical scavenging effect (%) = Ac – As / Ac × 100"

Where As = absorbance of test compound, Ac = absorbance of control

Reducing ability by the conversion of ferric into ferrous state

The reducing ability was determined by the conversion of ferric into ferrous state by antioxidant compounds using the method of Oyaizu (1986). Each test compound (100 µl: 10 to 1000 µM) prepared in DMSO was mixed with phosphate buffer (250 µl: pH 6.6: 0.2 M). Potassium ferricyanide (250 µl: 1%) was then added to the contents in the test tube. This mixture was then incubated at 50°C for twenty minutes in water bath and was centrifuged for ten minutes at 3000 rpm. Subsequently on centrifugation, the top layer of solute (250 µl) was separated in another set of test tubes and mixed with equal volume of DMSO (250 µl). Ferric chloride (0.1 %: 50 µl) was added to the mixture with absorbance at 700 nm on spectrophotometer (Specord 2000, Germany). Percent reduction ability was determined in terms of percentage with respect to BHA used as standard.

Percent reduction activity = At/As × 100

Where As = absorbance of standard, At = absorbance of test.

Table 1. *In-vitro* antioxidant activity of Linkus formulation including lozenges and Syrup w.r.t. Standard.

S\N	Concentration Tested (µg/ml)	Percent Activity (%) (syrup) ± SEM	Percent activity ± SEM (%) (Lozenges)	Percent activity ± SEM (%) (standard)
1	10	33.4±0.9464	21.2±0.421	71.2± 0.41
2	50	45.7 ± 0.6454	40.7 ± 0.443	87.9± 0.45
3		65.0±0.9124	58.0±0.512	96.8± 0.51

Table 2. Linkus lozenges and syrup reducing ability w.r.t. Standard.

S\N	Concentration tested (µg/ml)	Percent Activity (%)(syrup) ± SEM	Percent Activity (%)(capsules)) ± SEM	Percent Activity (%) (standard) ± SEM
1	10	12.8 ± 0.312	10.3 ± 0.131	43.7 ± 0.421
2	50	34.5 ±0.412	24.7± 0.213	65.8 ± 0.321
3	100	43.6± 0.561	34.6 ± 0.312	87.4 ± 0.112

Antiulcer/anti urease activity

By using the indophenol method, the urease activity was evaluated by ammonia production as described by Weatherburn. Reaction mixtures encompassing 25 µl of enzymatic (Jack bean Urease) solution and 55 µl of buffers comprising 100 mM urea were incubated with 5 µl of test compounds for 15 min at 30°C in 96-well plates (Tariq et al., 2011). With the help of indophenol method, activity was determined by measuring ammonia production. After that, 45 µl of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µl of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added with each well. By using a microplate reader (Molecular Device, USA), the absorbance at 630 nm was measured after 50 min. In a final volume of 200 µl, all reactions were performed in triplicate. By using SoftMax Pro software (Molecular Device, USA), the results (change in absorbance per min) were processed. All the assays were performed (0.01 M $K_2HPO_4.3H_2O$, 1 mM EDTA and 0.01 M $LiCl_2$) at pH 8.2. Percentage inhibitions were intended from the formula 100 - ($OD_{testwell}$ / $OD_{control}$) × 100. As the standard inhibitor of urease and Thiourea was used (Khan et al., 2013).

Lipoxygenase inhibition activity

Lipoxygenases are family of iron encompassing dioxygenases that convert the addition of molecular oxygen to fatty acid comprising a cis-1, 4- pentadiene classification. The prime product of this response is a “4-hydroperoxycis trans-1, 3-conjugated pentadienyl moiety” within unsaturated fatty acid. This assay processes the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase with lionoleic acid as substrate (Tappel, 1986: Chedea et al., 2012). In the proposed method, lipoxygenase enzyme solution was prepared in sodium phosphate buffer with such concentration to give 130 U per well. Sodium phosphate buffer (pH 8.0: 160 µl:100 mM) was occupied in each well of plate labelled as Blank named B *substrate* and B *enzyme*, as control and Test. Test compound solution in methanol (10 to 1000 µM: 10 µl) was added in each well labelled as test. Lipoxygenase solution (LOX: 20 µl) was added in each well including B *enzyme,* Control and Test except B *substrate* and the mixture was incubated at 25°C for ten minutes. Substrate solution was prepared by adding linoleic acid (155 µl:0.5 mM) into 0.12% w/v tween 20 (257 µl). The mixture was mixed and 0.6 ml NaOH (1 N) was added to remove turbidity and volume was made up to 20 ml with deionized water. This mixture was dispersed with the nitrogen gas to evade autoxidation before adding to each other. The response was started by the adding of 10 µl substrate in each well except enzyme B, also the absorbance was measured at 234 nm for 5 min.

RESULTS

Linkus is the poly herbal formulation analyzed for antioxidant, reducing, and lipoxygenase and ACE inhibition activity with different concentration (10, 50, 100 µl/ml) on 2 dosage of different dosage forms, comprising lozenges and syrup. When formulations of syrup and lozenges were compared at various concentrations (10, 50,100 µg/ml), DPPH radical scavenging activity increased in a dose dependent manner for both formulations just like standard BHA as shown in Table 1. It showed that both dosage forms, including syrup and lozenges have good antioxidant potential that is, 23.4, 45.7, 65.0% w.r.t standard BHA. For determining the reducing activity, ferrous were the leading component. Both dosage forms of syrup and lozenges had some reducing ability as compared to standard as shown in Table 2. For protecting the gastric mucosa, the syrup and lozenges have some anti-urease activity too as shown in Figure 3 and Table 3. Lipoxygenase compounds are the derivatives of arachadonic acid. After analysis, it was determined that the lozenges and capsules have some efficacy for the reduction of inflammation. Results are shown in Table 4.

DISCUSSION

Oxidant cause damage to proteins, macromolecules and DNA and this causes many damages in human tissues

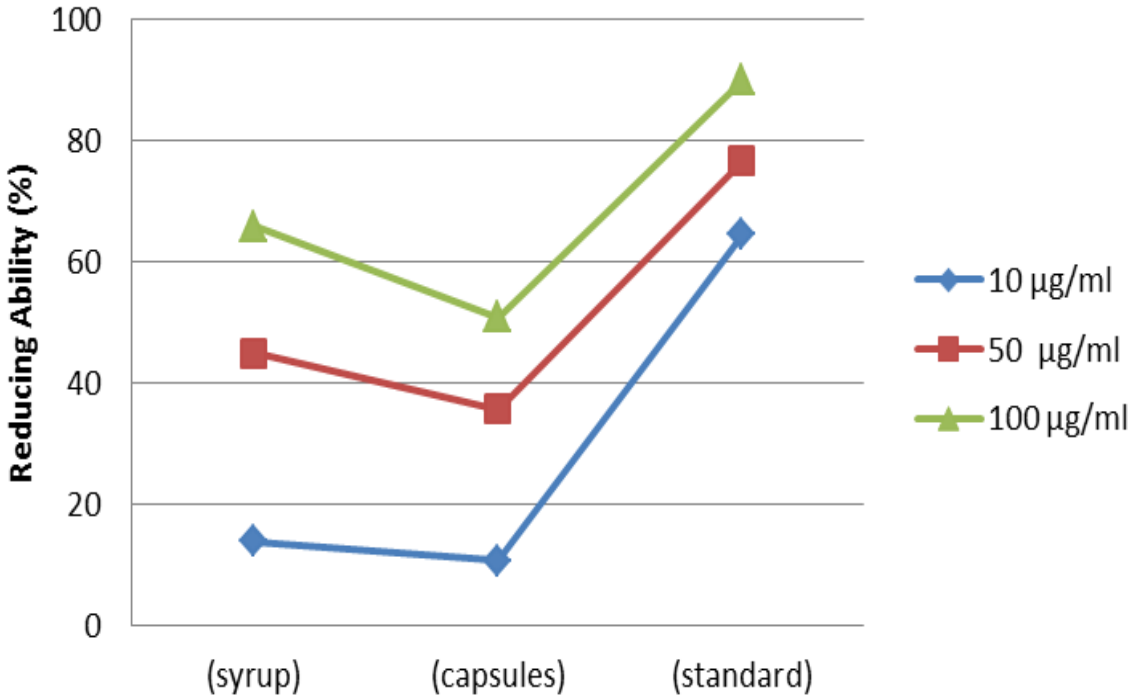

Figure 3. Antiurease activity of linkus lozenges and syrup w.r.t. standard.

Table 3. Antiurease activity of Linkus lozenges and syrup w.r.t. Standard.

S\N	Concentration tested (µg/ml)	Percent activity (%) (syrup)±SEM	Percent activity (%)(capsules)±SEM	Percent activity (%)(standard)±SEM
1	10	13.9±0.121	10.8±0.210	64.5±0.321
2	50	44.9±0.321	35.7±0.412	76.5±0.213
3	100	65.9±0.213	50.8±0.312	89.9±0.312

Table 4. Lipoxygenase inhibiting activity by poly herbal formulation linkus.

S\N	Concentration tested (µg/ml)	Percent Activity (%)(syrup) ± SEM	Percent Activity (%) (capsules) ± SEM	Percent Activity (%)(standard)) ±SEM
1	10	14.9 ± 0.213	12.2 ± 0.312	64.5± 0.410
2	50	46.8 ±0.611	31.0± 0.410	76.5± 0.612
3	100	56.1± 0.412	40.8 ± 0.312	89.9 ± 0.712

including aging (Ames et al., 1992; Fraga et al., 1990; Harman, 1981; Sai et al., 1992; Stadtman et al., 1992; Harman, 1992). Currently, available antioxidant compound including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have negative impact on human health (Barlow, 1990; Branen, 1975). For reducing the impact of oxidants, the natural occurring plants have been used for medical purpose (Schuler, 1990). Various plant species have been explored for antioxidant activity (Chu et al.,, 2000; Koleva et al., 2002; Mantle et al., 2000; Oke and Hamburger, 2002). DPPH is the sensitive method for antioxidant screening for plant extracts (Koleva et al., 2002). For determining the antioxidant activity in poly herbal formulation, scavenging

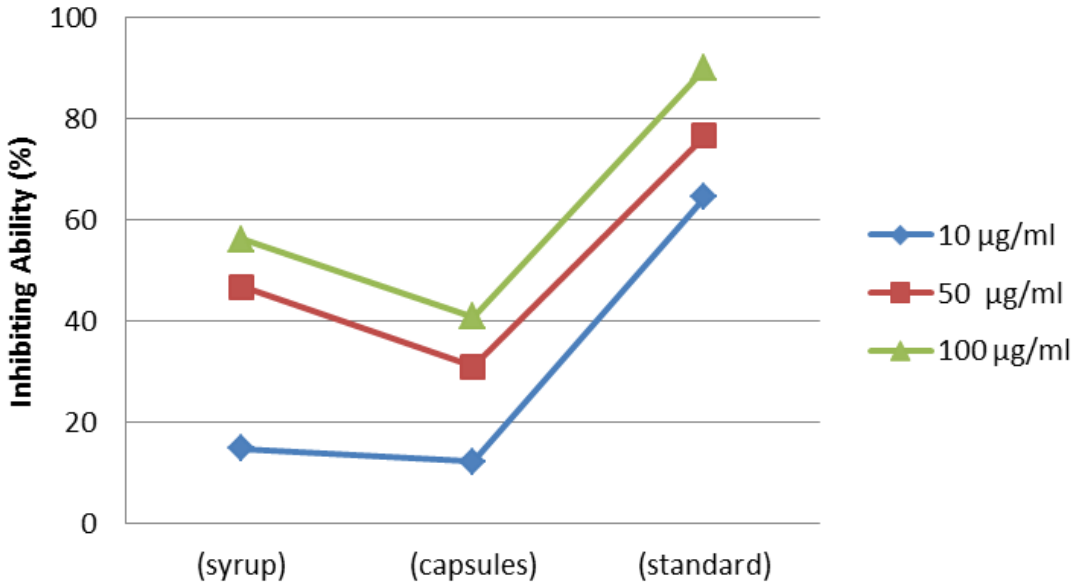

Figure 4. Lipoxygenase inhibiting activity by polyherbal formulation linkus.

ability was used. Absorption was noted on 517 nm due to odd electron. Good antioxidant activity was observed in both dosage forms. For determination of reducing activity Fe^{+3} to Fe^{+2} was the investigating point (Oyaizu, 1986). For antioxidant activity, the decreasing potential of a compound seems to be a substantial indicator (Meir et al., 1995). Percentage reducing ability was determined by the BHA standard formula and the results found good reducing ability in the poly herbal formulation.

Free radical and lipid per oxide play a vital role for the development of ulcer in human (Gutteridge, 1995). Linkus poly herbal formulation shows a good gastric protection. Herbal formulations have anti-inflammatory activity, including many disorders such as cough, chronic laryngitis and many others (Kapoor, 2000; Madras, 1993). Beside all the functions and activities linkus formulation shows good lipoxygenase inhibition activity (Figure 4).

Cough is the furthermost common today's symptom seen in overall family practice. Clinically, a cough is nearly a symptom of an underlying illness. It is significant to look beyond it to treat the cause and, hence achieve the maximal relief from cough and related symptoms. Studies have shown that there is an interaction between respiratory tract infections and antioxidant activities (Rubin et al., 2004; Gilliland, 2003) and oxidative stress present in blood due to respiratory infections (Gilliland et al., 2003). Lipoxygenase and leukotriene are the key factors for the inflammatory responses and respiratory distress (Wasserman et al., 1991). Multiple events suggested that lipoxygenase have strong relation in physiological event in respiratory tract infection (Holroyde, 1981; Weiss, 1982; Barne, 1984; Smith, 1985; Adelroth, 1986).

This study has shown the visible antioxidant activity, urease and lipoxygenase activity in poly herbal formulation Linkus cough syrup and lozenges (Figure 4). These dosage forms have contributing factors towards the indication cough as antioxidant and anti-inflammatory activity. These type of activity are due to free radical 2,2'-diphenyl-1-picryl hydrazyl, conversion of ferric into ferrous state, ammonia production using the indophenol method and hydroperoxides produced in the lipoxygenation reaction.

Conclusion

The poly herbal extract based lozenges and syrup were analyzed *in vitro* for anti-oxidant, urease and ACE inhibiting activity. Syrup was found to be more potent in comparison with lozenges but significant ability was found in contrast assessment with standard. It might be helpful for the reduction of respiratory tract infection and allied problems with minimum adverse/side effects.

Conflict of Interest

The authors have not declared any conflict of interest.

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REFRENCES

Adelroth E, Morris MM, Hargreave FE, O'Byme PM (1986). Airway responsiveness to leukotrienes C4 and D4 and to methacholine in patients with asthma and normal controls. N. Engl. J. Med. 315(8):480-484.

Ames BN, Shigenaga MK (1992). Oxidants are a major contributor to aging. Ann. N.Y. Acad. Sci. 663:85-96.

Barlow SM (1990). Toxicological aspects of antioxidants used as food additives. In Food Antioxidants, Hudson BJF (ed.) Elsevier, London. pp. 253-307.

Barnes NC, Piper PJ, Costello JF (1984). Comparative effects of inhaled leukotriene C4, leukotriene D4, and histamine in normal human subjects. Thorax 39(7):500-504.

Batt DG (1992). 5-Lipoxygenase inhibitors and their anti- inflammatory activities. Prog. Med. Chem. 29(1):439-449

Branen AL (1975). Toxicology and biochemistry of butylated hydroxyanisol and butylated hydroxytoluene. J. Am. Oil Chem. Soc. 5(2):59-63.

Chu YH, Chang CL, Hsu HF (2000). Flavonoid content of several vegetables and their antioxidant activity. J. Sci. Food Agric. 80(5):561-566.

De Souza MCR, Marques CT, Dore CMG, da Silva FRF, Rocha HAO, Leite EL (2007). Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. J. Appl. Psychol. 19(2):153-160.

Finkel T, Holbrook NJ (2000). Oxidants, oxidative stress and the biology of ageing. Nature 408(6809):239-247.

Ford-Hutchinson AW (1991). Potential and therapeutic value of development of 5-lipoxygenase inhibitors. In Leukotrienes and their products ed. Crooke ST, Wong A, San Diego: Academic Press. pp. 137-160.

Ford-Hutchinson AW, Gresser M, Young RN (1994). 5- Lipoxygenase. Ann. Rev. Biochem. 63:383-417.

Fox AJ, Lalloo UG, Belvisi MG, Bernareggi M, Chung KF, Barnes PJ (1996). Bradykinin-evoked sensitization of airway sensory nerves: a mechanism for ACE-inhibitor cough. Nat. Med. 2(7):814-817.

Fraga CG, Shigenaga MK, Park JW, Degan P, Ames N (1990). Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc. Natl. Acad. Sci. USA 87(12):4533-4537.

Ganapathy PS, Ramachandra Y, Rai SP (2011). In vitro antioxidant activity of Holarrhena antidysenterica Wall. methanolic leaf extract. J. Basic Clin. Pharm. 2(4):175-178.

Ghous T, Akhtar K, Nasim FUH, Choudhry MA (2010) .Screening of selected medicinal plants for urease inhibitory activity. Biol. Med. 2(4):64-69.

Gilchrist NL, Richards AM, March M, Nicholls MG (1989). Effect of sulindac on angiotensin-converting enzyme inhibitor-induced cough. Randomized placebo-controlled double-blind cross-over study. J. Hum. Hypertens. 3(6):451-455.

Gilliland FD, Berhane KT, Li YF, Gauderman WJ, McConnell R, Peters J (2003). Children's lung function and antioxidant vitamin, fruit, juice, and vegetable intake. Am. J. Epidemiol. 158:576-584.

Gülçin I, Berashvili D, Gepdiremen A (2005). Antiradical and antioxidant activity of total anthocyanins from Perilla pankinensis decne. J. Ethnopharmacol. 101(1):287-293.

Gutteridge JM (1995). Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin. Chem. 41(12):1819-1828.

Gutteridge J, Halliwell B (2000). Free radicals and antioxidants in the year 2000: a historical look to the future. Ann. NY Acad. Sci. 899(1):136-147

Gyamfi MA, Yonamine M, Aniya Y (1999). Free-radical scavenging action of medicinal herbs from Ghana: Thonningia sanguinea on experimentally-induced liver injuries. Gen. Pharmacol. Vasc. Syst. 32(6):661-667.

Halliwell B (2001). Role of free radicals in the neurodegenerative diseases. Drugs aging 18(9):685-716

Halliwell B (2008). Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch. Biochem. Biophys. 476(2):107-112.

Halliwell B (1994). Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 344(8924):721-724

Harman D (1981). "The aging process." Proc. Natl. Acad. Sci. 78(11):7124-7128.

Harman D (1992). "Free radical theory of aging." Mut. Res. DNAging 275(3-6):257-266.

Holroyde MC, Altounyan REC, Cole M, Dixon M, Elliott EV (1981). Bronchoconstriction produced in man by leukotrienes C and D. Lancet 318(8236):17-18.

Kapoor LD (2000). CRC hand book of Ayurvedic medicinal plants, (CRC Press, USA). p. 2.

Khan H, Saeed M, Muhammad N (2013). Lipoxygenase and urease inhibition of the aerial parts of the Polygonatum verticillatum, Toxicol. Ind. Health 2013 Apr 3:0748233713483197.

Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva LN (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal. 13(1):8-17.

Kuhnan J (1976). The flavonoids. A class of semi-essential food components; their role in human nutrition. World Rev. Nutr. Dietetics, 24:117-191.

Kumpulainen JT, Salonen JT (1999). Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease. The Royal Society of Chemistry, UK pp. 178-187.

Lin Cc, Yin MC (2007). B vitamins deficiency and decreased anti-oxidative state in patients with liver cancer. Eur. J. Nutr. 46(5):293-299.

Mantle D, Eddeb F, Pickering AT (2000). Comparison of relative antioxidant activities of British medicinal plant species *in vitro*. J. Ethnopharmacol. 72(1):47-51.

Mcmillan RM, Walker ERH (1992). Designing therapeutically effective 5-lipoxygenase inhibitors. Trends Pharmacol. Sci. 13:323-330.

Meir S, Kanner J, Akiri B, Hadas SP (1995). Determination and involvement of aqueous reducing compounds in oxidative defence systems of various senescing leaves. J. Agric. Food Chem. 43:1813-1815

Morice AH, Lowry R, Brown MJ, Higenbottam T (1987). Angiotensin converting enzyme and the cough reflex. Lancet 330(8568):1116-1118.

Nabati F, Mojab F, Habibi-Rezaei M, Bagherzadeh K, Amanlou M, Yousefi B (2012). Large scale screening of commonly used Iranian traditional medicinal plants against urease activity. DARU J. Pharm. Sci. 20(1):1-72.

Oke JM, Hamburger MO (2002). Screening of some Nigerian medicinal plants for antioxidant activity using 2, 2- diphenyl- picryl- hydrazyl radical. Afr. J. Biomed. Res. 5:1-2.

Oyaizu M (1986). Studies on product of browning reaction prepared from glucose amine Eiyogaku zasshi. Japan. J. Nutr. 44:307-315.

Rice-Evans CA, Miller NJ, Paganga G (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol. Med. 20(7):933-956.

Rubin RN, Navon L, Cassano PA (2004). Relation of serum antioxidants to asthma prevalence in youth. Am. J. Respir. Crit. Care Med. 169(3):393-398.

Sai K, Takagi A, Umemura T, Hasegawa R, Kurokawa Y (1992). Changes of 8-hydroxydeoxyguanosine levels in rat organ DNA during the aging process. Official organ of the International Society for Environmental Toxicology and Cancer. J. Environ. Pathol. Toxicol. Oncol. 11(3):139-143.

Schuler P (1990). Natural antioxidants exploited commercially, In Food Antioxidants, Springer Netherlands. pp. 99-170.

Smith U, Greenberger PA, Patterson R, Krell RD, Bernstein PR (1985). The effect of inhaled leukotriene D4 in humans. Am. Rev. Respir. Dis. 131:368-372.

Stadtman ER (1992). "Protein oxidation and aging." Science 257(5074):1220-1224.

Tariq SA., Ahmad MN, Obaidullah KA., Choudhary, MI, Ahmad W, Ahmad M. (2011). Urease inhibitors from Indigofera gerardiana Wall. J. Enzyme inhibit. Med. Chem. 26(4):480-484.

Wasserman MA, Smith III EF, Underwood DC, Barnette MA (1991).

Pharmacology and pathophysiology of 5- lipoxygenase products in Leukotrienes and their Products ed. Crooke ST, Wong A, San Diego: Academic Press. pp. 1-50.
Weiss JW, Drazen JM, Coles N (1982). Bronchoconstrictor effects of leukotriene C in humans. Science 216(4542):196-198.
Younes M (1981). Inhibitory action of some flavonoids on enhanced spontaneous lipid peroxidation following glutathione depletion. Plant Med. 43(11):240-245.
Zeeshan AS, Aqib Z, Saleha SK, Khan U (2014). Design, Development and Phytochemical Evaluation of a Poly Herbal Formulation Linkus Syrup. Chinese Med. 5:104-112.

Appendix 1. Linkus Syrup composition per 10 ml.

S.No	Ingredients	Quantity/10 ml
01	Adhatoda vasica – Bansa	600.00 mg
02	Piper longum – Filfil Daraz	100.00 mg
03	Cordia latifolia – Sapistan	100.00 mg
04	Glycyrrhiza glabra – Mulethi Extract	75.00 mg
06	Alpinia galangal – Khulanjan	50.00 mg
07	Viola odorata – Banafshan	25.00 mg
10	Onosma bracteatum – Gaozaban	100.00 mg
11	Methyl Paraben	10.928 mg
12	Propyl Paraben	2.168 mg
13	Sugar	7000.0 mg
14	Citric acid	20.00 mg
15	Glycerin	0.100 ml
16	Peppermint Oil	0.003748 ml
17	Clove Oil	0.001252 ml
18	Propylene Glycol	0.001668 ml

Appendix 2. Composition of Linkus Lozenges per lozenges.

S. NO.	Composition	Content (%)
1	Adhatoda vasica Nees.	1.2
2	Glycyrrhiza glabra L.	0.28
3	Piper longum L.	0.24
4	Hyssopus officinalis L.	0.12
5	Alpinia galanga (L.) Wild	0.12
6	Viola odorata L.	0.08
7	Mentha piperita L.	0.08
8	Sugar	q.s 100
9	Liquid glucose	40
10	Anhydrous citric acid	1.28
11	Talc	0.48
12	Mineral oil	0.176
13	Menthol	0.16
14	Eucalyptus Oil	0.12
15	Paraffin	0.04
16	Beeswax	0.04
17	Lanolin	0.04
18	White soft paraffin	0.024
19	Tablet essence for Orange lozenges	0.852
20	Tablet essence for Honey lemon lozenges	0.852