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RESEARCH ARTICLE

Bioactive Molecule of Onion Leaves Act as Inhibitor of PPO.

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ABSTRACT:
Bioactive molecules of onion leaves exhibited potent anti polyphenol oxidase (PPO) activity; which was purified on silica gel by using solvents. Purification profile procured 3 and 2 active butanol and methanol fractions respectively. Reticence of PPO activity was observed within range of 12 to 14 min when it was treated with active fractions. Characterization of it was conceded by physical constant, UV, IR and TLC. It reputes that, active hydroxyl group attached to the ring structures of PPO, which quench free radical by donating hydrogen in the active hydroxyl group of PPO, confirming inhibition of pigmentation reaction in vitro. Bioactive molecules from onion leaves exhibited various types of inhibition against oxidation of L-DOPA and L-tyrosine and it was compared with classical inhibitors of PPO. Significance of bioactive molecules is to explore for drugs designing, understanding of ligand–receptor interactions, environmental problems and it is simple alternative for the inhibition of PPO.

KEYWORDS: Onion leaves; quercetin; oxazolone; flavanoid; cinnamic acid; inhibition.

INTRODUCTION:
Onion and onion leaves is essential ingredient of vegetarian and non-vegetarian diets having medicinal and nutritive value. Though onion is useful, onion leaves turn black when cut, due to oxidation of 3, 4-dihydroxyphenylalanine (dopa) to pigmented products. This reaction is catalyzed by group of enzymes; polyphenol oxidase (PPO). Pigmentation is often an undesirable reaction, which is responsible for unpleasant sensory qualities, significant loss in nutritional qualities and market value of fruits/vegetables. The prevention of this reaction has always been a challenge to food scientist[1]. Discrimination of this reaction is responsible for various types of diseases and disorders viz Parkinsonia[2], albinism**,** melanoma, psychological effect-schizophrenia[3,4], gastrointestinal side effects and melanin-based bias in human societies.

In India, Maharashtra is the major place where fruits and vegetable are cultivated on a large scale but in peak season if transport facilities become unavailable, fruits and vegetable being perishable, leads to economic loss to the farmers. Consequently change in color is a major problem. Many inhibitors are often used to prevent the pigmentation, which are toxic to human beings.

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Therefore searching for nontoxic inhibitors having broad application for these activities is important in developing a rational chemotherapy of pigmentation. In the present paper, the biologically active compounds from onion leaves extracted by using organic solvent were bioassay for inhibitory effect. Bioactive conformation of drugs is one of the key points for understanding the ligand–receptor interactions[5]. Therefore, the Onion leaves organic extract was purified on silica gel. Fractions were characterized by physical constant, TLC, UV and IR spectra.

MATERIALS AND METHODS:
Materials:
L-dopa, l-tyrosine, sodium phosphate (mono and dibasic), ammonium sulfate, citron X-100, EDTA, ascorbic acid were obtained from E. Merck (India). Sephadex G-25 from pharmacia, silica gel ammonium hydroxide, di-ethylether , chloroform, acetone from Loba Chemicals, butanol from SRL. Other chemicals used were of analytical pure grade. Methanol was obtained locally and was distilled prior to use.

Source:
Fresh onion leaves were obtained directly from the fields, were washed and then stored at 4°C in a refrigerator for about 24 hrs.

PPO extraction from onion leaves by solvent and salt method:
Fresh onion leaves (300 g) were homogenized with 150 ml of ice-cold acetone, water with Citron X-100 (80:19:1,

V/V) for about 24 hrs (-18°C). After filtration through a muslin cloth the residue was washed 3x with 150 ml of acetone (-18°C). The resulting acetone powder was dried for about 30 min. at room temperature and was suspended in 200 ml of 0.05 M phosphate buffer pH 7.0, containing 0.01mM EDTA, and 0.1mM ascorbic acid. Sonicated for 1 hr. and then centrifuged at 5600 *g* for 15 min. Solid ammonium sulfate was added to 60 % molar saturation, the solution was stirred for about 3 hrs. and kept for about 24 hrs. at 4°C, centrifuged at 5600 *g* for 30 min. The resulting pellet was homogenized in 50 mM Sodium phosphate buffer pH 7.0 and Dialyzed for about 20 hrs against the same buffer at 4°C. Monophenol monooxygenase, o-diphenol oxidase activity and protein were measured at each stage. After partial purification, a sample containing 0.687gm of protein with 7700IU activity for monophenol monooxygenase and 1900 IU activity o-diphenol oxidase was loaded on affinity column using natural affiant lignin.

Extraction and Isolation of inhibitor of PPO by organic solvent method:

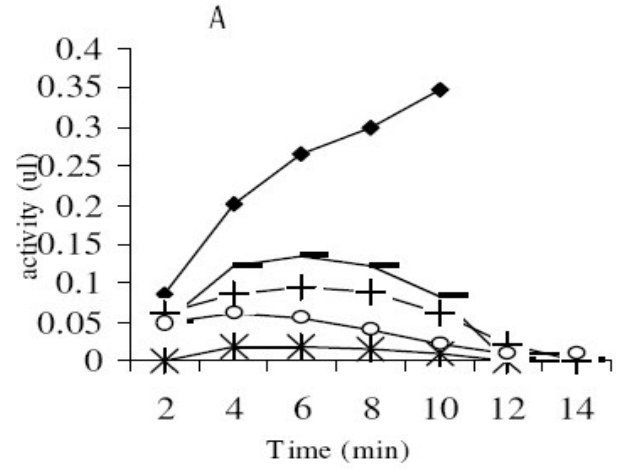
The dried onion leaves (1Kg) were extracted with butanol and methanol separately at room temperature, sonicated for 3 hrs, concentrated and dried under reduced pressure. Fifty gram of butanol extract and methanol extract was subjected to 100 ml chloroform and NH_4OH: water (1:2) separately. It was compared with water extracts. The butanol fraction (3.2g) and methanol fraction (2.0g) was loaded on silica gel column (Loba, Mesh 60-120 5.2g of silica ,height 9cm and diameter–2cm) and successively eluted with gradient of diethyl ether and cyclohexane (1:1). 12 to 14 active fraction of butanol were pooled and re-chromatographed on silica gel column. While 6 to 9 active fraction of methanol were pooled and re-chromatographed. By increasing polarity of eluting solvent it procured 3 major fractions for butanol extract and two major fractions for methanol extract. Characterization of these fractions was carried out qualitatively by TLC, UV and IR and quantitatively by aluminum chloride method[6], vitamin C by iodometric method[7].

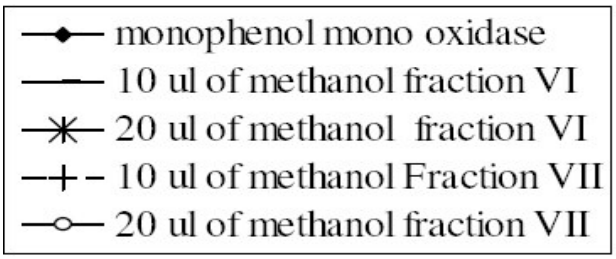
Effect of inhibitor on monophenol monooxygenase and o-diphenol oxidase:

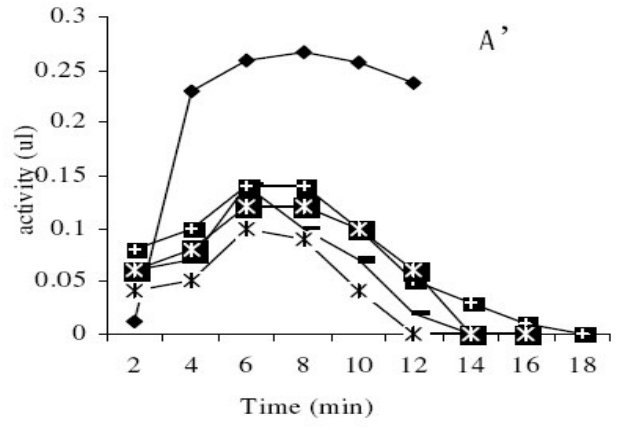
Monophenol monooxygenase and o-diphenol oxidase was isolated from onion leaves on natural column. Various concentrations of pure active fractions were incubated with 10 µl of PPO separately. Isolated inhibitors were compared with classical inhibitors, Viz. benzoic acid and cinnamic acid and endogenous inhibitor. Inhibition of monophenol monooxygenase and diphenol oxidase was measured by spectrophotometrically method and by TLC.

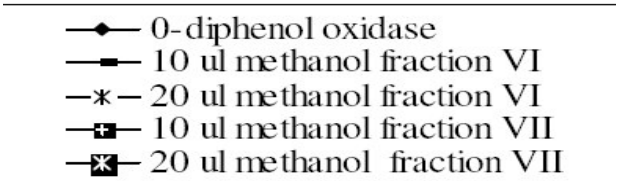
RESULTS AND DISCUSSION:

Extraction of onion leaves with organic solvent followed by re-chromatographed on silica gel column, that procured three active fractions for butanol extract and two active fractions for methanol extract. When 10µl and 20 µl of methanol fractions and butanol fractions were incubated with 10µL monophenol monooxygenase and o-diphenol oxidase activity separately, Total inhibition of activity was observed at 12 and 18 mins. respectively for methanol active fractions (fig 1 A and A') while 14 mins. for butanol fractions (fig. 1 B and B').









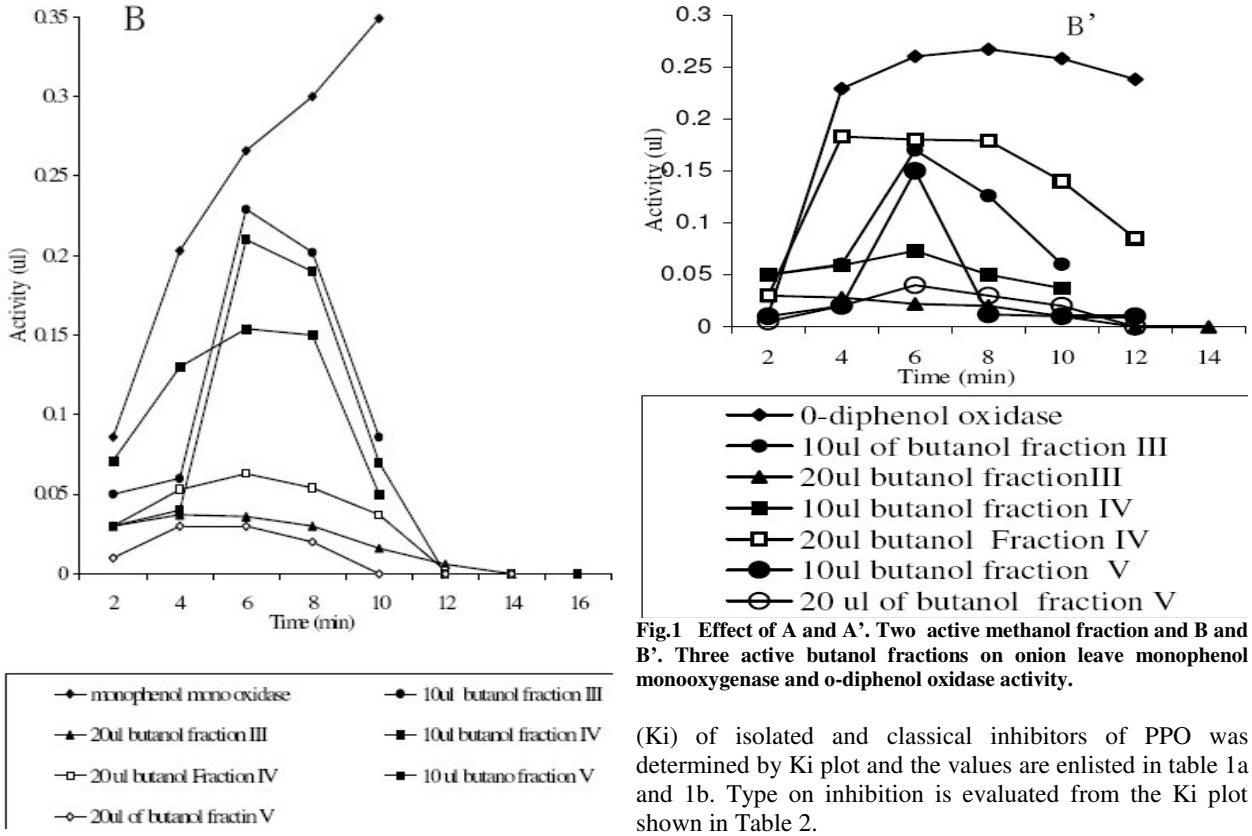


Fig.1 Effect of A and A'. Two active methanol fraction and B and B'. Three active butanol fractions on onion leave monophenol monooxygenase and o-diphenol oxidase activity.

(Ki) of isolated and classical inhibitors of PPO was determined by Ki plot and the values are enlisted in table 1a and 1b. Type on inhibition is evaluated from the Ki plot shown in Table 2.

Table 1 a): Ki values for isolated inhibitors from onion leaves of PPO.

Isolated inhibitor from onion leaves Free and Immobilized PPO		**Ki values monophenol monooxygenase**		**Ki values of o-diphenol oxidase**	
				Free	Immobilized
Biochemical method	Fraction I	-0.13	-0.14	-0.19	-0.20
	Fraction II	-0.10	-0.10	-0.22	-0.20
Organic method	Fraction III	-0.09	-0.08	-0.11	-0.11
	Fraction IV	-0.10	-0.10	-0.11	-0.11
	Fraction V	-0.23	-0.22	-0.14	-0.15
	Fraction VI	-0.14	-0.13	-0.10	-0.10
	Fraction VII	-0.14	-0.14	-0.10	-0.12

Free Immobilized under the heading of Ki values monophenol monooxygenase (same as in o-diphenol oxidase)

Table 1b): Ki values for classical inhibitors of PPO

Ki values classical inhibitors onion leaves Free and Immobilized PPO							
monophenol monooxygenase				o-diphenol oxidase			
Benzoic acid		Cinnamic acid		Benzoic acid		Cinnamic acid	
Free	Immobilized	Free	Immobilized	Free	Immobilized	Free	Immobilized
-0.20	-0.19	-0.53	-0.49	-0.23	0.22	-0.11	-0.12

Table no:2 Type of inhibition:

	Fraction I (endogenous inhibitor)	II (endogenous inhibitor	III	IV	V	VI	VII
Free monophenol monoxygenase	Competitive	Competitive	Non-Competitive	Non-competitive	Mixed	Mixed	Mixed
Immobilized monophenol monoxygenase	Mixed	Mixed	Mixed	Mixed	Mixed	Non-Competitive	Non-Competitive
Free o-diphenol oxidase	Mixed	Mixed	Non-Competitive	Non-competitive	Mixed	Mixed	Mixed
Immobilized o-diphenol oxidase	Competitive	Competitive	Non-Competitive	Non-competitive	Mixed	Mixed	Mixed

Inhibitory actions of these bioactive fractions gave insights to characterize it for structural exhibition. In view of it, attempt were made to emphasized the structural elucidation of bioactive fractions by TLC, UV and IR that are is follows-

Methanol fraction VI :(98 mg) MP=140^{0}C Rf =0.91, UV= 201 nm, IR = 3437.39(N-H stretching) cm^{-1}, 2142.78cm^{-1}, 1655.23cm^{-1} (C-O aromatic ketone), 1456.81cm^{-1}(C=C stretch), 1246.41cm^{-1} (C-O stretch), 1110.72cm^{-1} (C-O stretch), 759.65cm^{-1}(C-H stretch).

Methanol Fraction VII:(99.56 mg),M.P= 316^{0}C Rf = 0.84, UV= 211.80 nm IR= 3441.58 cm^{-1} (H bonded alcohol), 2954.93 cm^{-1} (C-H stretch), 2924.73 cm^{-1}(Aromatic C-H stretching), 2852.49 cm^{-1} (C-H stretch) , 1732.47 cm^{-1} (C=O stretching), 1465.63cm^{-1}(C=C stretch), 1382.75 cm^{-1}, 1367.05 cm^{-1}, 1215.26 cm^{-1}, 1168.45 cm^{-1}, 1045.08 cm^{-1}(C=O stretch), 874.47 cm^{-1}(C-H stretch),760.98 cm^{-1}(C-H stretch), 588.94 cm^{-1}.

Butanol Fraction III: (100 mg) MP= 316^{0}C Rf = 0.79, UV= 391.60 nm IR = 3410.70(OH stretch) cm^{-1}, 2926.20 (–H aliphatic) cm^{-1}, 1642.63(C=N stretch) cm^{-1}, 1464.86 (C=C) cm^{-1}, 1217.05(C-O stretch) cm^{-1}, 761.39 (Aromatic C-H bending) cm^{-1}.

Butanol Fraction IV: (102 mg) MP=140^{0}C Rf = 0.67, UV= 377.60 nm IR = 3392.22 cm^{-1} (-OH stretched), 2925.53 cm^{-1}, 2134.82 cm^{-1} (-C≡C- stretch), 1638.82 cm^{-1}(C=N stretching), 1464 cm^{-1} (-CH_2 Stretch), 1216.17 cm^{-1} (C-O aldehyde), 761.39 cm^{-1} (C-H bend).

Butanol Fraction V: (103 mg) MP=316^{0}C Rf = 0.89, UV= 351.40 nm, IR = 3465.44 cm^{-1}(OH stretching), 2922.16cm^{-1}(C-H stretching), 2851.06cm^{-1}(C-H stretching), 1715.63cm^{-1}(C-O aldehyde),1463.79cm^{-1} (C=C stretching), 1192.84cm^{-1} (C-C stretching),1047.91 cm^{-1},758.07 cm^{-1} (C=C) stretching is observed out of plane bonding. Vibrations are at 758.07 cm^{-1}.

All above spectroscopic as well as physical constant results may conclude that, methanol fraction VI and butanol fraction IV closely resembles oxazolone derivative structure while methanol fraction VII and butanol fraction III and V matches quercetin structure. Inhibition of onion leaves PPO is due to quercetin and derivative of oxazolone. Literature data also noted that the derivative of oxazolone[8] and quercetin[9] inhibits the enzyme activity. Khalid et. Al[9] demonstrated that oxazolone derivative showed excellent in-vitro tyrosinase inhibitory properties. Onion leaves contains 2.15 ± 0.04 % of flavanoid as these fractions were flavonoid in nature[10]. Vitamin C is a cofactor of tyrosine pathway and responsible for coloration. 12.20 % per 100 mg vitamin C present in onion leaves which induced modulation of skin immune function in human beings and in fruits and vegetables. Therefore, this study leads to development of new drugs for the rational chemotherapy of pigmentation.

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