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STUDY ON INHIBITORY EFFECT OF *ROSMARINUS OFFICINALIS* L EXTRACTS AND QUERCITINE ON PARTIALLY PURIFIED COW'S BRAIN POLYAMINE OXIDASE

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ABSTRACT : This study involved the recognition of polyamine oxidase PAO in cow's brain extract, using dialysis and DEAE-cellulose ion exchanger chromatography. The enzyme was partially isolated and the purification fold of just onepeak (I) obtained was 17.71. Rosemary (*Rosmarinus officinalis* L.) for this study was analyzed. The phytochemical compounds: Flavoniod, Alkaliod, Glycoside, Terpine, Saponin, Taninewere obtained by according to suitable extraction protocols. Using HPLC technique, some polyphenolic compounds were determined. It is noted the presence of Quercitine, Luteolin and Apegeninein macerated and soxhlet extracts of leaves, while absence of Catechin and Rutine. Macerated and soxhlet extracts of twigs has been observed the presence of both Quercitine and Catechin.

The inhibitory effect of *Rosmarinus officinalis* L. extracts on PAO I activity was studied. The macerated extract of twigs with 1 mg/ml and Soxhlet extract of leaves with 4 mg/ml showed 100% inhibition. Lineweaver-Burk plots show a non competitive inhibition of PAO by using pure Quercitine. Km value was 35.71 mM, which represent K'm value with inhibitor. V max value is variable from 0.185 unit/ml without inhibitor to 0.138 and 0.107 unit/ml with 4 and 6 mM of quercitine as inhibitor, respectively. Accordingly, inhibition constant Ki values were 5 and 7 mM, respectively. Our study is aimed to investigate the polyphenolic composition of rosemary in Mosul, to reveal the affluence of these compounds and assess the inhibitory effect of its extracts.

Key words : Polyamine oxidase, rosemary (*Rosmarinus officinalis* L.), cow's brain, quercitine.

INTRODUCTION

Polyamines (PAs), which have potent biological activity are aliphatic compounds havingtwo or more amino groups (Vuosku *et al*, 2018; Chen *et al*, 2019). Both eukaryotic and prokaryotic cells are widely containing these molecules (Liu *et al*, 2017 and Mustafvi *et al*, 2018). In living organisms, these small molecules mainly existin free orconjugated forms (Gholami *et al*, 2013). Polyamines, spermidine and spermine serve numerous brain-specific functions. Polyamine transport mechanisms may account for the redistribution of these organic cations, which may also be synoptically released as neuromodulators or neurotransmitters, in the brain (Laube *et al*, 2014). Therefore, PAs are key elements of many diseases and syndromes (Serguei *et al*, 2014). Spermidine and spermine are accumulated in glia and their distribution is clearly evolutionarily determined; it is found throughout the brain (Laube and Veh, 1997) retina (Biedermann *et al*, 1998 and Skatchkov *et al*, 2000), peripheral nervous system (Lindquist *et al*, 1985) and in glial-neuronal co-cultures (Gilad *et al*, 1999) of multiple species, including man (Biedermann *et al*, 1998). PA catabolism is finely regulated by polyamine Oxidase (PAO) (Cervelli *et al*, 2018), which catalyzes the oxidation of PA and acetylpolyamines. Members of the PAO gene family have been recognized in a wide variety of plants and fungi as well as animals, including vertebrates, arthropodes, placozoa. Spermine, N1-acetylspermine and N1-acetylspermidine can be oxidized by yeast PAO (Polticelli *et al*, 2012 and Pegg, 2016).

Cells can be protected against damage of reactive oxygen species by antioxidant compounds, which are capable of delaying, retarding or preventing oxidation (Valko *et al*, 2007; Moon *et al*, 2019). Ramalakshmi *et al*

(2009) indicated that phenolic compounds are antioxidants, which interact with free radical species and are consumed during the reaction. The biological properties of secondary metabolites, polyphenols are widely spread in plants and known for their beneficial health effects (Koechlin-Ramonatxo, 2006). Among the inventory of medicinal plants from Mosul, Iraq. The herb, rosemary (*Rosmarinus officinalis* Lamiaceae) is growsin the Mediterranean basin. Owing to its different usesas a common domestic cooking spice for flavoring, this plant is cultivated worldwide. Moreover, the extracts of this herb have been broadly used as an additive in food stuff process because of their latent high antioxidant activity (Sotelo-Félix *et al*, 2002). It has been formerly described that isolated components from rosemary extracts exhibit inhibitory effects on the growth of breast, liver, lung and leukemia cancer cells (Djeridane *et al*, 2006 and Bricha *et al*, 2010).

These potent biological activities have been referred to the occurrence of several bioactive compounds in its composition. The main families exist in rosemary are phenolic diterpenes including: carnosic acid, carnosol or rosmanol; flavonoids such as genkwanin, cirsimaritin or homoplantaginin and triterpenes as ursolic acid (Bai *et al*, 2010 and Del Baño *et al*, 2004).

MATERIALS AND METHODS

Plant collection : The harvesting of *Rosmarinus officinalis* L. samples is conducted in full bloom in March from nursery of Mosul University, Mosul, Iraq. The aerial parts, leaves and twigs free from diseases were collected in the early morning, rinsed in water, darkness dried and powdered using blender.

Purification of PAO from cow's brain : The crude extraction of cow brain, which was obtained from slaughter house has been optimized in our lab to produce a maximum yield. Briefly, water extraction was conducted using 50 gm of brain in 150 ml water, then freeze by liquid nitrogen, shake in ice bath and centrifuged at 3000 g for 5 min, the supernatant was collected and stored to purify.

Step I : Dialysis : In dialysis tube 10 ml of crude extract was placed and magnetically stirred against 20 mM phosphate buffer, pH 7.2 overnight at 4^0C, during dialysis the buffer was altered every 6 hrs (Robyt and White, 2001).

Step II: Ion Exchange Chromatography : 10 ml of dialyzed enzyme solution was applied on DEAE-cellulose anion exchanger column, following by 20 mM phosphate buffer, pH 7.2. The protein was eluted at a flow rate 0.8ml/min. The protein was detected by following the absorbance at 280 nm. PAO activity was measured in the fractions,then pooled and lyopholized.

PAO assay : PAO activity was determined spectrophotometrically in an automated shimadzu UV-1800 UV Spectrophotometer by method of Dahel *et al* (2001) using spermine as substrate. The decrease in absorbance at 410 due to the reduction of potassium ferricyanide as electron acceptor was followed. One unit of PAO activity was defined as that amount of enzyme which oxidized 1 micromole of spermine per min.

Protein determination : Total protein conc. was estimated at 650nm, using BSA as a standard (Scharcterle and Pollack, 1973).

Macerated extraction (ME) : The ethanolic extraction of rosemary has beenoptimized in our lab to produce a maximum yield. Briefly, 100g of both leaves and twigs were maceratedusing in 600 ml ethanol(95%), at room temperaturefor 24hrs, then filtered by medical gauze and centrifuged at 3000 rpm for 15 min. The supernatants were concentrated using rotary evaporator and dried in the desiccator to obtain crude extract (Rebiai *et al*, 2014).

Soxhlet extraction (SE) : For extraction, the powdered materials 50g/500 ml was subjected to soxhlet extraction and exhaustively extracted with petroleum ether for 48 hours at 60-80^0C for deffating. The extracts were evaporated using rotary evaporator. The batch was macerated in ethanol (95%) for 24 hrs, extracted by soxhlet for 6 hrs and evaporated (Sharma and Janmed, 2014).

Detection of some active compound in dry plant and alcoholic extracts : The presence or absence of the flavonoids, alkaloids, terpens, glycosides in dry plant and alcoholic extracts can be detected by method of Senguttuvan *et al* (2014) and Zeghab (2013). Saponins and Tanines can be detected by method of Harbon (1984).

HPLC analysis : HPLC analysis is performed on a Trcerextrasil ODS1 C-18 column (250 × 4.6 mm), 5 µm particles size, an aliquot (50 µl) was injected and eluted at the temperature of 40°C according to the Muchuweti *et al* (2007), using solvent acetonitrile – water (80:20, v/v) as the mobile phase. At wavelength 280nm, analysis was accomplished at a flow rate of 1 mL/min. Inmethanol, standards solutions were prepared with the range of 10-40 ppm. The degassing and filtering through 0.45 µm membrane filter (Millipore) of samples, standards solutions and mobile phase were processed. The mobile phase was acidified to ensure the total protonation of the compounds studied. The analyzed using a Prominence Auto-Sampler (SIL-20A) equipped with Shimadzu LC-

20AT (Shimadzu, Kyoto, Japan) reciprocating pumps connected to a DGU-20A5 degasser and CBM-20A integrator.

PAO inhibition : From stock solution 1% (1gram of leaves and twigsalchoholic extracts in 100 ml DW), different concentrations (1,2,3,4,5 mg/ ml) and quercitine with 4and 6 mM were prepared. 0.2 ml of of PAO was incubated with 0.1 ml of extract as inhibitor at 37^{0}C for 30 min. The activity was measured at 410 nm using spermine as substrate (Beffani *et al*, 2001).

RESULTS AND DISCUSSION

Purification of PAO : The results of PAO purification profile were tabulated in Table 1. One peak of PAO was purified to about 17.71 fold compared to crude enzyme with specific activity 6.34 U/mg protein, respectively. Fig. 1 shows that PAO elution started from 115- 155 ml.

Two peaks of PAO were purified from sheep brain with specific activity 3.876 and 2.856 U/mg protein (Ali and Younis, 2019). This finding is consistent with what researchers found (Touhala, 2006; Al-Lehebi, 2013). The increase in the specific efficacy of PAO can be attributed to the disposal of low molecular weight compounds such as amino acids, peptides and ions that can surround the active site and act on enzyme efficacy.

This indicates that the purified PAO carries the result of negative charge opposite to the ion exchanger charge. These results were identical to those obtained for purified PAO from mother's milk, two distinct peaks each of had PAO activity, showed the specific efficacy of 17.36 and 13.02 unit/mg protein, respectively (Al-Katib, 2000) as well as matching PAO purified from cerebrospinal fluid

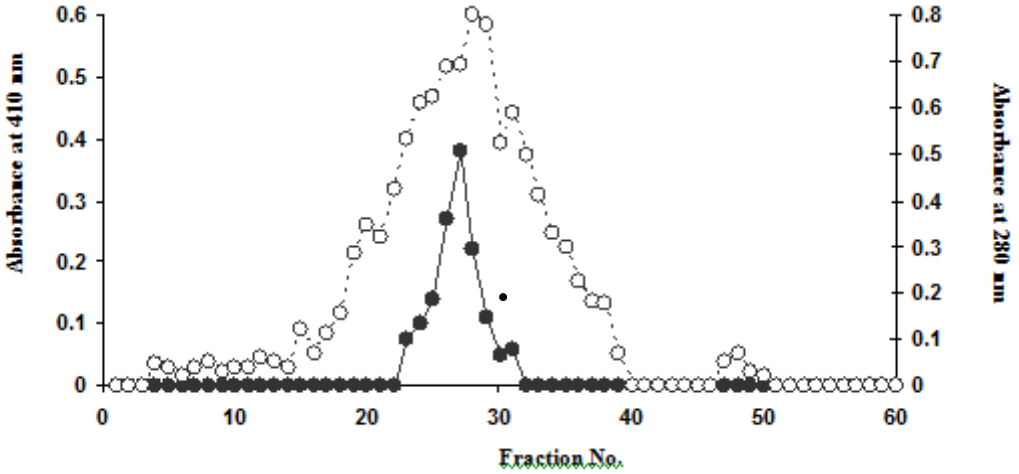


Fig. 1 : Elution profile of PAO purification by DEAE-cellulose chromatography column (40×2.5 cm). PAO activity at 410nm●, Protein at 280nm .

Table 1 : PAO purification steps from cow brain.

Purification steps	Volume (ml)	Total protein (mg)	Total activity U*	Specific activity (U/mg protein)	Yield %	Purification fold
Crude	80	137.92	49.92	0.36	100	1
Dialysis	8	10.58	23.88	2.25	47.83	6.25
Ion exchange						
Peak I	45	4.34	27.54	6.34	55.18	17.71

*:A unit is defined as amount of PAO, which convert one micromole of spermine per minute at 25^{o}C.

Table 2 : Detection of some active compounds in dry plant and extracts of *Rosmarinus officinalis* L. leaves and twigs.

Active compounds	Dry plant	ME of leaves	SE of leaves	ME of twigs	SE of twigs
Flavoniod	+	+	+	+	+
Alkaliod	+	+	+	+	+
Glycoside	+	+	+	+	+
Terpine	+	+	+	+	+
Saponin	+	+	+	+	+
Tanine	+	+	+	+	+

Table 3 : Retention time and concentrations of phenolic compounds in leaves extracts of *Rosmarinus officinalis* L (µmol/ ml).

Standard compounds	Rt	ME		SE	
		Rt	µmol/ml	Rt	µmol/ml
Apegenine	4.12	4.30	46.5	3.76	106.5
Catechin	11.9	—	—	—	—
Quercitine	6.56	6.63	30	6.63	34.5
Kampferol	8.21	—	—	8.64	84
Luteolin	5.18	5.34	106	5.53	28
Rutine	7.25	—	—	–	–

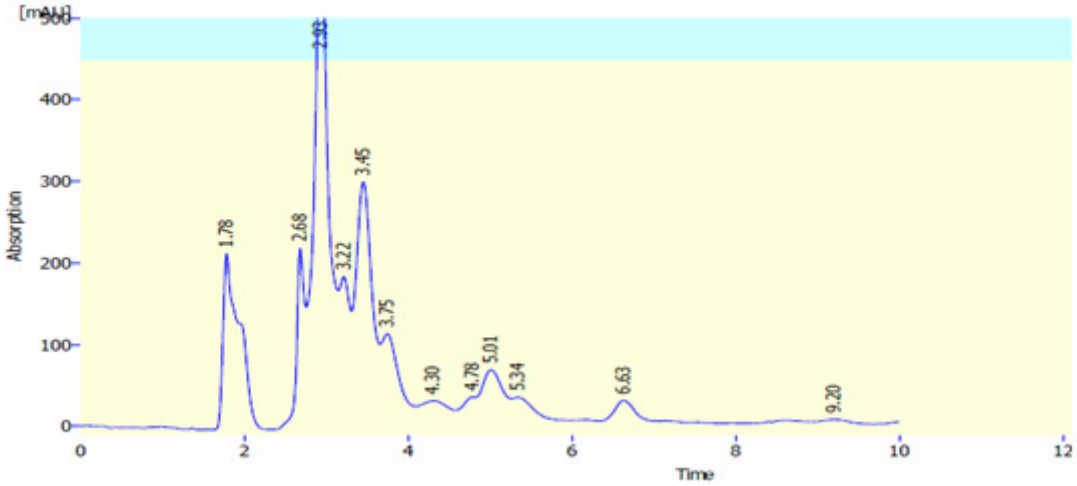


Fig. 2 : Identified polyphenolic compounds in macerated extract of *Rosmarinus officinalis* L. leaves by HPLC.

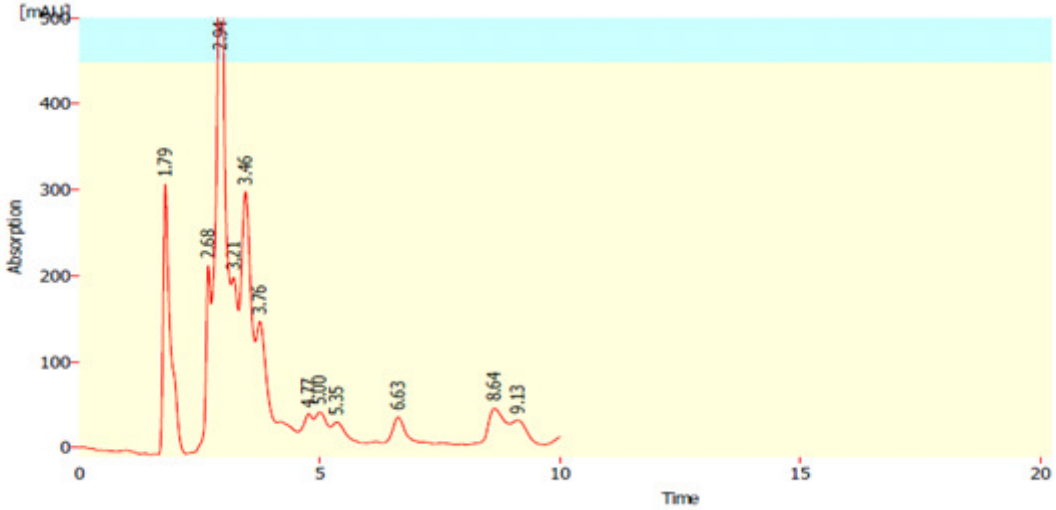


Fig. 3 : Identified polyphenolic compounds in Soxhlet extract extractof *Rosmarinus officinalis* L. leaves by HPLC.

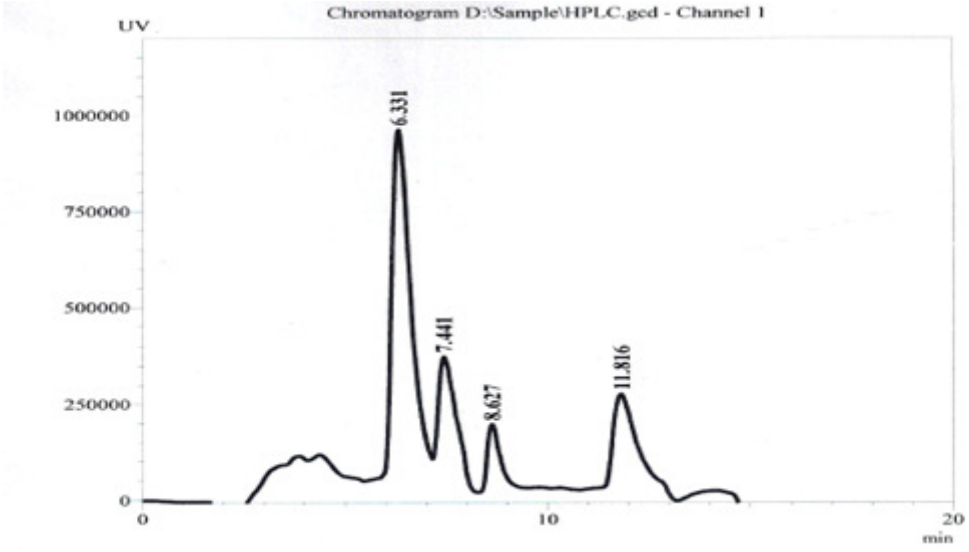


Fig. 4 : Identified polyphenolic compounds in macerated extract of *Rosmarinus officinalis* L. twigs by HPLC.

of children with two distinct peaks each of which has the efficacy of 1201.92 and 1157.22 unit/mg protein respectively (Touhala, 2006), while three distinct peaks of PAO from red blood corpuscles appeared for healthy women with efficacy of 0.194, 0.182 and 0.0984 unit/mg protein, respectively (Al-Lehebi, 2013).

Detection of chemical group : Using identification

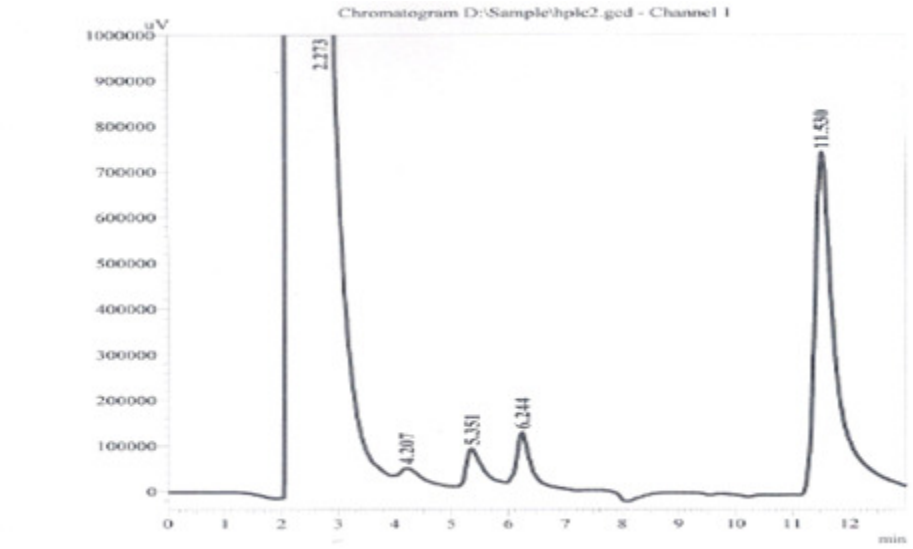


Fig. 5 : Identified polyphenolic compounds in Soxhlet extract of *Rosmarinus officinalis* L. twigs by HPLC.

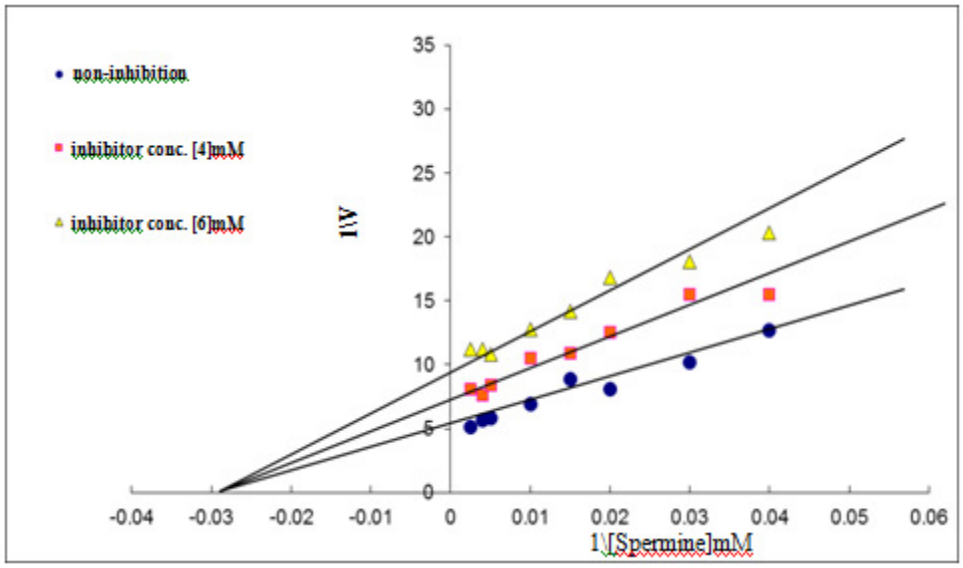


Fig. 6 : Lineweaver-Burk plot shows inhibition type on partially purified PAO activity by quercitine.

methods for activated chemical groups in leaves and twigs of *Rosmarinus officinalis* L. The results indicated presence of some active compounds such as Flavoniod, Alkaliod, Glycoside, Terpine, saponin and Tanine (Table 2). These results are according to studies of Salih *et al* (2015), who have observed the presence of these active compounds in plant. Investigation of presence of active compounds (Flavonoids, Alkaloids, Tannins, Saponine, Resine and Glycoside) in same plant was carried out (Abdullah, 2010).

HPLC analysis : Using HPLC, some polyphenolic compounds were determined in alchoholic extracts after acid hydrolysis. It is noted the presence of Quercitine (30 and 34.5 µmol/ ml), Luteolin (106 and 28 µmol/ ml) and Apegenine (46.5 and 106.5µmol/ml) in macerated and Soxhlet extracts of leaves respectively, while absence of Catechin and Rutine (Table 3, Figs. 2 and 3).

Table 4 : Retention time and concentration of phenolic compounds in twigs extracts of *Rosmarinus officinalis* L. (µmol/ml).

Standard compounds	Rt	ME		SE	
		Rt	µmol/ml	Rt	µmol/ml
Apegenine	4.12	—	—	4.20	0.106
Catechin	11.9	11.81	206.7	11.53	50.72
Quercitine	6.65	6.33	112	6.24	0.091
Kampferol	8.21	8.62	68.8	—	—
Luteolin	5.18	—	—	5.35	0.24
Rutine	7.25	7.44	52.64	—	—

As them acerated and Soxhlet extracts of twigs have been observed the presence of both Quercitine (112 and 0.091 µmol/ ml) and Catechin (206.7 and 50.72 µmol/ ml) respectively (Table 4, Figs. 4 and 5). UV-visible spectrophotometry analysis revealed that the main contents of total polyphenols and flavonoids are gained in the methanol extract by decoction (Haida *et al*, 2015).

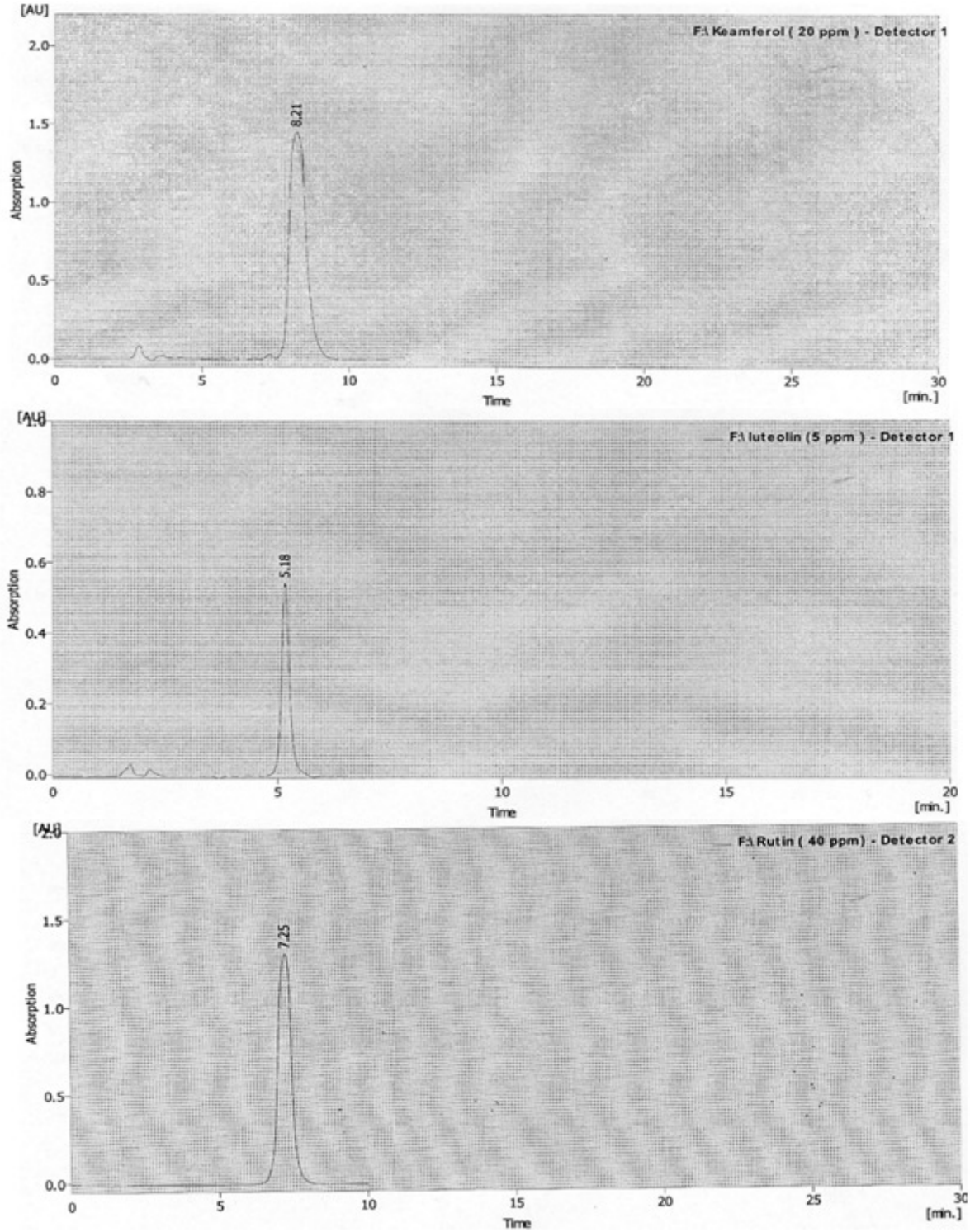
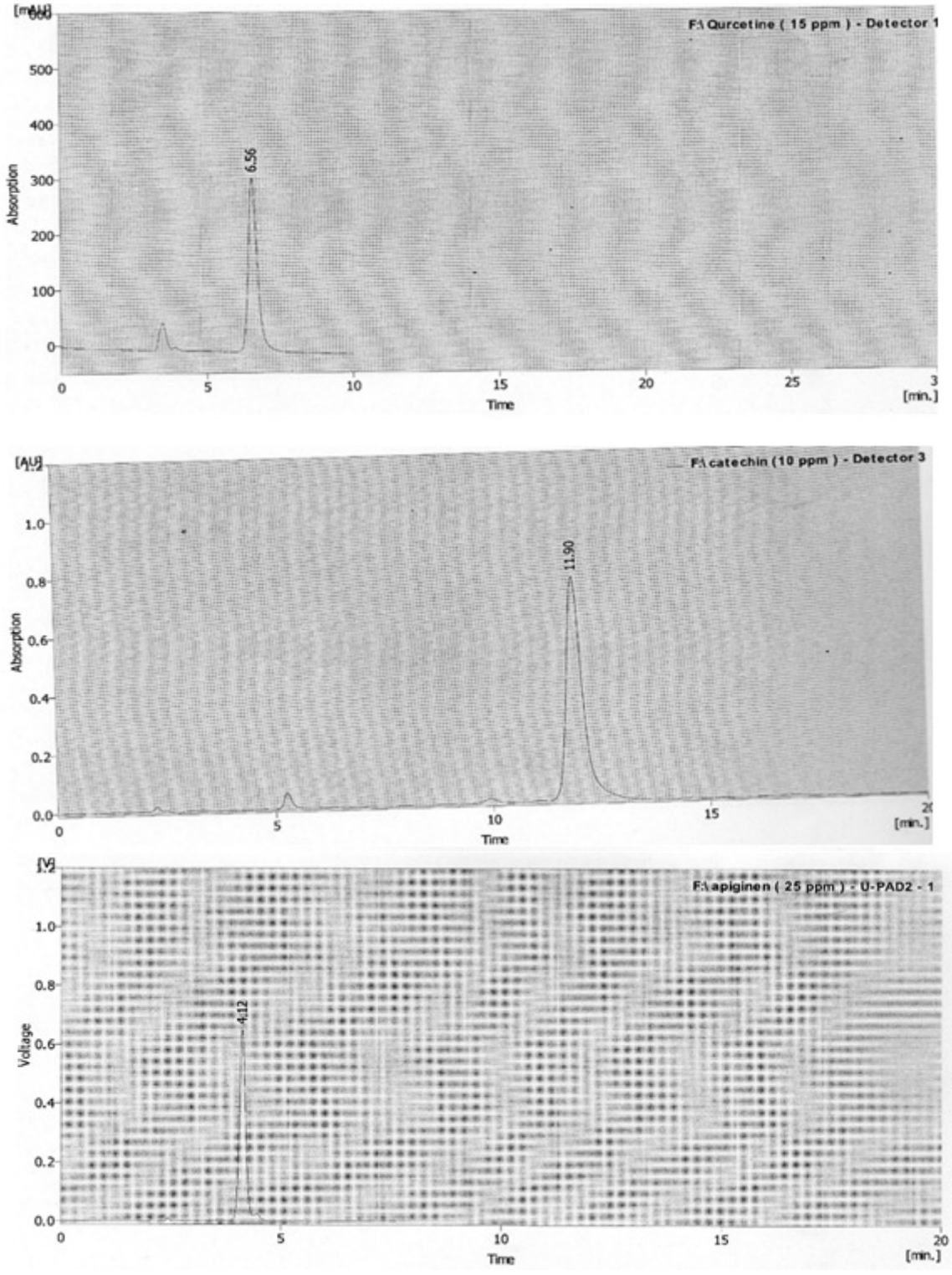


Table 5 : Effect of alcoholic extracts of *Rosmarinus officinalis* L leaves and twigs on PAO activity.

Alcoholic extracts conc. (mg/ml)	Inhibitory effect of leaves ME (%)	Inhibitory effect of leaves SE (%)	Inhibitory effect of *twigs* ME (%)	Inhibitory effect of *twigs* SE(%)
Control	0.00	0.00	0.00	0.00
1	43.7	68.8	100	81.3
2	62.4	12.5	75	62.4
3	25	6.3	12.5	68.8
4	49.9	100	12.5	18.7
5	12.5	37.6	6.2	6.2



Rosemary biological properties have been recognized to rich composition with phenolic compounds. However, the positive contribution of flavonoids to rosemary bioactivity is also reported in the literature (Sasaki *et al*, 2013 and Afonso *et al*, 2013). Furthermore, the phenolic composition has been observed to vary depending on agronomical and processing circumstances (Almela *et al*, 2006; Ribeiro *et al*, 2016).

Effect of *Rosmarinus officinalis* L. extracts on PAO activity : Table 5 shows the inhibitory effect of leaves and twigs *Rosmarinus officinalis* L. alchohilic extracts on partially purified PAO activity with different concentration. The complete inhibition (100%) obtained at 4 mg/g of leaves by Soxhlet extract. However, the complete inhibition (100%) obtained at 1 mg/g by macerated extract of twigs *Rosmarinus officinalis* L.

Lineweaver-Burk plots (Fig. 6) shows a noncompetitive inhibition of PAO by using Quercitine. Km value was 35.71mM, which represent K'm value with inhibitor. Vmax value is variable from 0.185 unit/ml without inhibitor to 0.138 and 0.107 unit/ml with 4 and 6 Mm of quercitine as an inhibitor, respectively. Accordingly, inhibition constant (Ki) values were 5 and 7mM, respectively.

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