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Evaluation of the effect of *Castanea sativa* extracts on lipoxygenase activity

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Abstract

Lipoxygenase LOX is a lipolysis enzyme that oxidises unsaturated fatty acids like arachidonic and linoleic acids to form unhealthy chemicals such as dienes, leukotrienes and malondialdehyde in advanced oxidation, which is harmful to cells. In this study, the polyphenols and saponins compounds were extracted from *Castanea sativa* using Soxhlet for 3 days. Colourimetric and HPLC techniques were used to identify polyphenols and saponins in the extracts respectively. The effects of these extracts were evaluated on LOX activity, which was purified from the liver of Iraqi Cows (AL-Sharabi Cows) as well as on *E. coli* and *Pseudomonas* bacteria resistance. According to the LOX purification procedures, the specific activity increased from 0.001 to 0.03 U/mg with a purification fold of 30 times and a yield percentage of 174. Gallic acid 7.72 mg, Rutin 29.25 mg, Quercetin 27.6 mg, kaempferol 34.42 mg, Apigenin 5.25 mg and Catechin 25.8 mg/100 gm of Castanea fruit was obtained. The inhibitory effect of polyphenol and saponin extracts on LOX activity was at 100 and 40 µg respectively. Line weaver-Burk plot was used to investigate the type of inhibition that was found non-competitive. However, these extracts were studied at concentrations of 500, 250 and 125 µg on bacteria resistance. Polyphenols had the best effect at 500 and 125 µg on *E. coli* and *Pseudomonas* bacteria respectively. Whereas, saponin had the best effect at 250 and 125 µg for *E. coli* and 125 µg for *Pseudomonas.*

Keywords

oxidative stress, polyphenol, saponin, inflammation, fatty acids

Introduction

Lipoxygenases (LOXs) are big monomeric protein that produces hydroperoxides by oxygenation of polyunsaturated fatty acids (PUFA) such as linoleic, linolenic and arachidonic acid (1, 2). LOXs are widely found in a diverse range of animals, plants and fungi (3). The 6 human lipoxygenases (LOX-5, LOX-12, LOX12B, LOX-15, LOX-15B and LOX-E3), as well as the molecules that create them, have been linked to genetic diseases (4). Furthermore, 5-LOX is found in all mammals and oxygenates carbon atom number C5 in arachidonate, whereas LOXs in plants catalyse the oxygenation of linoleic acids and linolenic acids at carbon atoms number C9 or C13 by 9-LOX, 13-LOX respectively, also conversion of fatty acids (1,4-pentadiene) to fatty acids having conjugated hydroperoxy (5, 6). In humans, lipoxygenase plays an important and essential role in stimulating inflammatory responses via the production of leukotrienes which are pro-inflammatory mediators and lipoxins, which are anti-inflammatory mediators (8).

Reactive oxygen species (ROS) levels that are too high might induce inflammation, producing cytokines and then activating LOXs. LOXs have been associated with several inflammatory disorders, including cancer, stroke, cardiovascular, neurological diseases, kidney failure, metabolic syndrome and skin diseases (7, 8).

LOXs are associated with human diseases and finding novel LOX inhibitors is a critical step in preventing disease (9). Many LOX inhibitors were used for therapeutic applications, such as Zileuton and Minocycline (10). These inhibitors have been banned or restricted due to their negative side effects (11). Nowadays, many bioactive compounds are derived from a plant. Phytochemicals perform a vital defence role in plants, which could be useful in preventing human disease (12). Many more useful natural compounds are still to be discovered (13).

Natural products have been used to treat various diseases. They are still a reliable source for many medications used today because they have a large number of secondary metabolites with a wide range of chemical structures and pharmacological actions (14).

The *Castanea sativa* has been used as a source of polyphenol and saponin compounds having a lot of bioactivities effects (15). Polyphenols are among the most abundant and widely distributed classes of secondary metabolites in plants. These compounds are of considerable interest because of their unique features. The primary sources of phenolic compounds are fruits, seeds, leaves, stems and peels (16). With over 8000 known phenolic structures, phenolic compounds are among the most common and widespread families of chemicals in the world of plants (17). These compounds are found practically in all plant parts and have a variety of roles based on their structure (18).

Polyphenols are secondary metabolites that are required for plant development and reproduction. They are also used as natural antioxidants, anti-hepatotoxic, anti-inflammation, inhibition of lipid peroxidation, carcinogenesis inhibition, antibacterial activity and vasodilatory activity (19). Furthermore, saponins are secondary metabolites derived from steroid or triterpenoid aglycone called genuine or sapogenin coupled by glycosidic linkage of one or more sugar molecules, and because of the presence of both sugar (polar) and steroid or triterpene (nonpolar) molecules saponins have a high surface-active capability which is responsible for many of their negative beneficial activities (20) and may interact with membrane and cellular components. For instance, Saponins show insecticidal, allelopathic, cytotoxic properties, and antimicrobial, together with antinutritional effects (21). Saponins also have been shown to inhibit digestive enzymes such as chymotrypsin and trypsin, as well as inhibition of protein degradation by generating saponin-protein complexes (22). However, specific saponins have been shown to have beneficial nutritional effects, such as hypocholesterolemia and improved growth in different types of animals (21).

This study aimed to detect the ability of polyphenols and saponin extracts from *Castanea sativa* for the inhibitory activity of lipoxygenase and evaluate their activity against bacterial resistance.

Materials and Methods

Preparation of the plant extracts

Turkish *Castanea sativa* was used in this study, Family: Fagaceae and Genus: Castanea (23).

500gm of Turkish *Castanea sativa* fruit was taken and homogenised after mixing with distilled water (1:3) weight : volume and then the crude extracts were prepared (24).

Isolation and identification of extracts

Polyphenol and saponin extraction

Polyphenols were extracted by Soxhlet for three days using ethanol 99% (25). Saponin was isolated from the remaining tissues after flavonoids extraction in the same way with distilled water by a Soxhlet for 3 days (26).

Initial identification of flavonoids was done by using the colourimetric method using a mix of 5 ml of flavonoid extract with 5 ml of solution (consisting of 10 ml 50 % ethyl alcohol + 10 ml 50% potassium hydroxide), the appearance of yellow colour indicates the presence of flavones (27). Phenolic compounds were detected by adding 3 ml of extract to 2 ml. of ferric chloride solution, and the appearance of a bluish-green colour indicates the presence of phenolic compounds (28). Saponin was identified by Dragendorff's reagents, which is a potassium bismuth iodide solution containing basic bismuth nitrate, tartaric acid and potassium iodide, which generates an orange or orange to red precipitate when in contact with alkaloids (29). HPLC-chromatography was used for the identification of the polyphenols in flavonoid extracts (30).

Extraction of lipoxygenase

In this research, the liver was chosen because it is the main tissue of fat metabolism and the LOX enzyme play a significant role in this process.

Liver source

Liver samples of cows were obtained freshly from a slaughterhouse and stored at -0^0C. Six different animals were sources of liver samples (31).

Crude extract preparation

250 gm of the liver was sliced and homogenised by homogeniser (Sorvall Dupont Instruments) by 0.02M phosphate buffer pH 7.6 (2 ml: gm) tissue, then the mixture was centrifuged (Heraeus-christ Gmbh) at 12000xg for 20 min at 4 ^{0}C, the pellets were neglected. 100 ml from the supernatant was saved as a crude extract for LOX purification (31).

Ammonium sulfate precipitation

The protein was precipitated from the crude extract by progressively adding solid ammonium sulfate saturated 60% (w:v) and stirred for 20 min. at 4 ^{0}C, leaving it overnight and then centrifuged for 30 min at 12000×g, finally dissolved in 20 ml buffer phosphate (32).

Dialysis

Protein obtained from the precipitation process was dialysed in a dialysis tube with a cutoff 10Kd with phosphate buffer in the water bath at 4 ^{0}C for 24 hr, changing buffer every 6 hr (33).

Diethylaminomethyl-Cellulose Chromatography

The sample obtained from dialysis was applied in column 40 2.5 cm containing DEAE-Cellulose gel with buffer phosphate pH 7.2, the elution was collected at about 1.2 ml/min (34).

Lipoxygenase assay

LOX activity was assayed by monitoring the increase in the absorbance at 234 nm by the change of the cis,cis-1,4 pentadiene of linoleic acid into the conjugated hydroperoxyldiaene derivative cis-trans- (2). The unit of the enzyme defines as the amount of enzyme that converts one µmol of the substrate (linoleic) to the product in a min (35).

Effect of polyphenols and saponin on LOX -activity

The effect of polyphenols and saponin were studied on LOX activity which was purified from Cow's liver and bacteria as shown below:

1. Determined the optimal inhibitory concertation of extracts: the concentrations of polyphenol extract were 50, 100, 200, 300, 400, 500, 600, 800 µg while saponin at concentrations 10, 20, 40, 60, 80, 100, 120, 140 µg was added to the above reaction LOX mixture, the inhibition percentage was calculated relative to the control activity without extracts (36).
2. The inhibition type was studied using the best inhibitory concentration of extracts with the different concentrations of substrate (linoleic).
3. Effect of polyphenols and saponin on *E. coli* and *Pseudomonas aeruginosa* bacteria resistance. Media bacteria culture was prepared for *E. coli* and *Pseudomonas* and was treated with three concentrations of polyphenols and saponin extracts at 500, 250 and 125 µg/ml. Area zone radius effects (inhibition) around bacteria spots were measured (37).

Results and Discussion

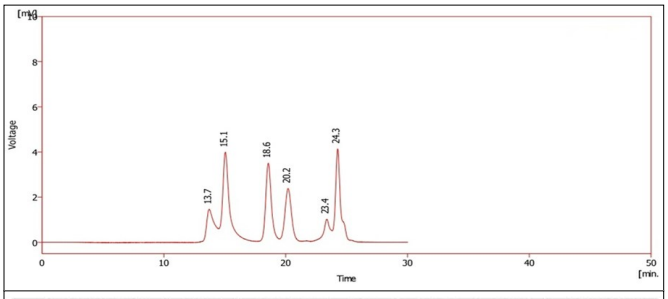
This study was designed to obtain natural products polyphenol and saponin and assess their effects on LOX activity and some kinds of bacteria.

Identification of Castanea sativa extracts

Flavonoids, polyphenol, and saponin were identified in extracts preliminary by colour tests. Polyphenol was specifically diagnosed in the HPLC technique and gallic acid, rutin, quercetin, kaempferol and apigenin were found in Fig. 1. The amount of these compounds was mg/100 gm of fruit as shown in Table 1.

Purification procedure of LOX

Linolic acid was used as a substrate for LOX activity detection because of its higher specificity than other fatty acids, such as Linolenic acid, Arachidonic acid, Eicosatetraenoic acid and Docosahexaenoic. The purification steps of LOX were shown in Table 2. Enzyme activity increased from 0.093U/ml to 0.18U/ml with a yield percentage of 174 and the specific activity increased from 0.001 to 0.03 U/mg protein with several



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	13.727	10.425	0.507	18.0	11.8	0.31	Gallic acid
2	15.050	15.635	1.107	26.9	25.7	0.22	Rutin
3	18.577	11.170	0.891	19.2	20.7	0.21	Qurcetin
4	20.203	10.443	0.683	18.0	15.9	0.27	Keamferol
5	23.377	4.320	0.336	7.4	7.8	0.23	Apigenine
6	24.270	6.033	0.778	10.4	18.1	0.14	Catechine
	Total	58.025	4.302	100.0	11.0		

Fig. 1. HPLC-Chromatogram of polyphenol extracts.

Table 1. Showed the amount and percentage of natural compounds in *Castanea sativa* fruit

Compounds	mg/100g	%
Gallic acid	7.72	5.90
Rutin	29.25	22.43
Quercetin	27.60	21.22
Kaempferol	34.42	26.41
Apigenin	5.25	4.24
Catechin	25.80	19.80

Table 2. The purification steps of LOX enzyme from cow's liver

	Volume	Total protein (mg)	Activity U/ml	Total activity U	Specific activity U/mg protein	Purification fold	Yield %
Crude	100	7109	0.093	9.3	0.001	1	100
$(NH_4)_2SO_3$	22	1709	0.45	9.9	0.005	5	106
Dialysis	21	1659	0.51	10.71	0.006	6	115
DEAE-cellulose	90	540	0.18	16.2	0.03	30	174

purifications folds 30, Fig. 2. It was also showed an increase in total and specific LOX activity in rat liver with one peak of LOX (29) and one peak with a specific activity of 0.4U/mg in Porcine Leukocyte LOX purification (38).

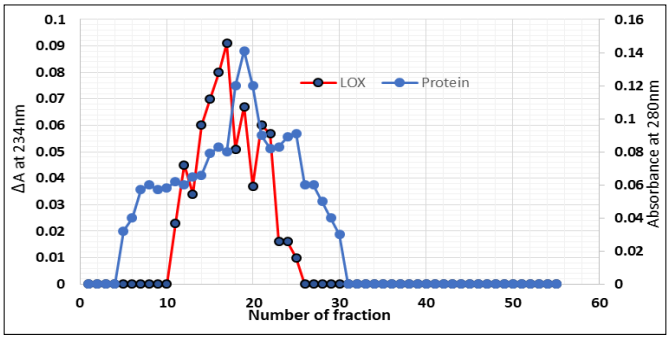


Fig. 2. Elution profile of LOX DEAE-cellulose purification from cow's liver.
U: The amount of enzyme that converts micromole of the substrate to products per minute.

The best inhibitory concentration

The results in Table 3 demonstrated the percentage inhibition calculated by the equation (38).

$$\text{inhibition} = \frac{[control - test] \times 100}{control}$$

Table 3. The best concentration inhibition of polyphenol and saponin

Polyphenol		Saponin	
Concentration µgm	% Inhibition	Concentration µgm	% Inhibition
50	42	10	49
100	48	20	43
200	45.5	40	53
300	44	60	48
400	41	80	40
500	40	100	50
600	36	120	22.5
800	33	140	39

The best inhibitory effect of LOX by polyphenol and saponin was illustrated in Fig. 3 and 4 at concentrations of 100 µgm and 40 µgm respectively (39).

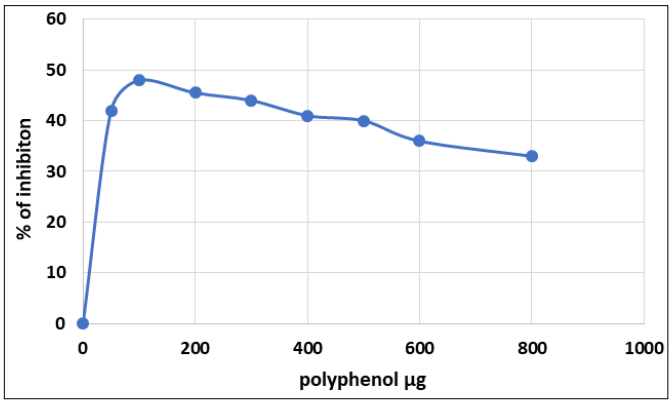


Fig. 3. Inhibition percentage of LOX by polyphenol extract.

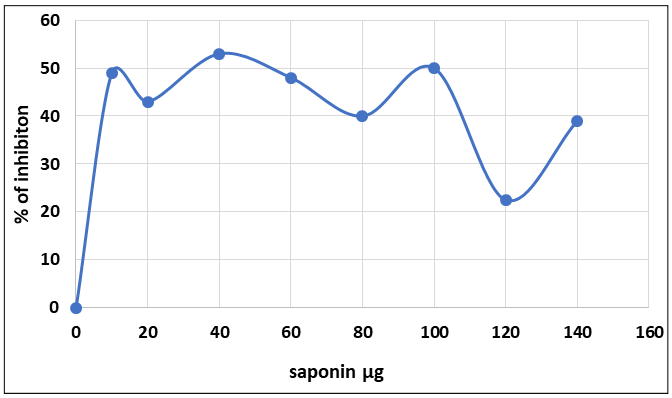


Fig. 4. Inhibition percentage of LOX by saponin extract.

Inhibition type of LOX activity

The effect of substrate (linoleic) was demonstrated in Fig. 5 and the inhibition type of LOX by applying line weaver-burke equation with polyphenol and saponin extracts at the best inhibition concentrations (35) was studied, the results showed a non-competitive inhibition type with two extractions. Fig. 6 and 7 demonstrated the non-competitive inhibition of extracts on LOX-activity. Kinetics variables were Maximum velocity (Vmax) 1.9 U/ml and Michaelis-Menten constant (km) 0.33mM without inhibitors (control) while with polyphenols Vmax 1.25 U/ml and with saponin Vmax 1.0U/ml, where no change in Michaels-Minton constant km 0.33mM as demonstrated in Table 4.

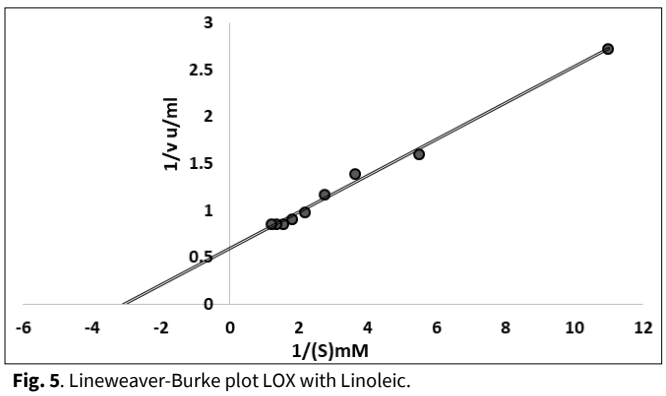


Fig. 5. Lineweaver-Burke plot LOX with Linoleic.

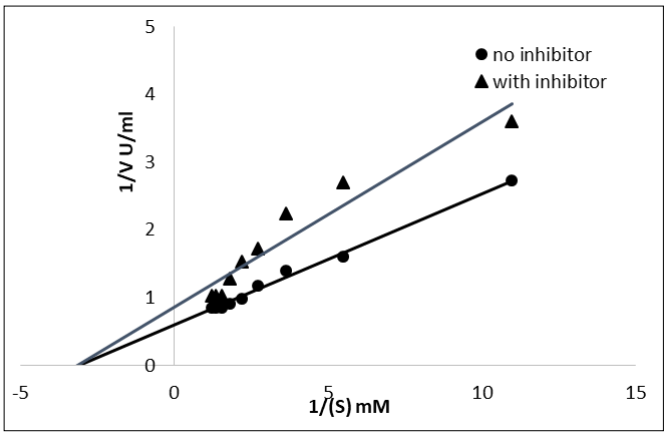


Fig. 6. Lineweaver-Burke plot LOX inhibition by Polyphenol extracts.

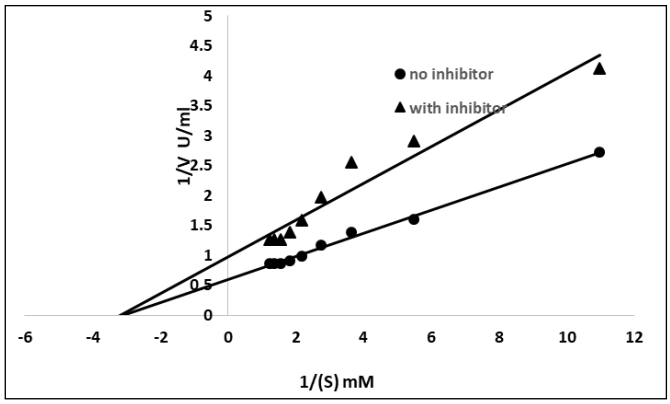


Fig. 7. Lineweaver-Burke plot LOX inhibition by saponin extracts.

Table 4. Inhibition type by polyphenol and saponin and kinetic variables

	The best concentration	Inhibition type	Vmax U/ml	Km mM
Control	No inhibitor	-	1.90	0.33
Polyphenols	100µg	Non-competitive	1.25	0.33
Saponin	40 µg	Non-competitive	1.0	0.33

Polyphenols and saponins have one or more hydroxyl groups or active groups in their structure and may be linked or interfere indirectly with amino acid residues or iron in the active sides in LOX (40, 41) and decrease or limit the activity. It was found that polyphenol extracts inhibition of the soy LOX because the same compounds that are associated or binding with LOX and their structure changed their effect on the one within the axis of the enzyme's action as a cofactor (40). LOXs are dioxygenases that catalyse the oxidation of polyunsaturated fatty acids like linoleic and arachidonic acids and produce hydroperoxides. Immune, epithelial and tumour cells all express LOX enzymes which have a wide range of physiological activities, including skin disease, inflammation and carcin-

ogenesis (42). Leukotrienes are important inflammatory mediators produced by the 5-LOX. However, this enzyme requires the presence of the 5-LOX activating protein in intact cells (FLAP) (43).

Effect of extracts on Bacteria resistance

Escherichia coli (*E. coli*) is a highly adaptable bacteria that can vary from healthy to unhealthy in the gut (44). Within a few hours of birth, the gastrointestinal system is colonised by commensal *E. coli*. Numerous clinical reports revealed that *E. coli* has been related to the aetiology of diarrhoea in people and their companion animals (45). Even though these strains are a common part of the human and animal microbiome. Three concentrations of 500, 250 and 125 µg/ml of each polyphenol and saponin were applied to *E. coli* and *Pseudomonas aeruginosa* media and detected their effects using area radius coloured measurement around the bacteria wall.

The results in Table 5 and Fig. 8 show the effect of polyphenol and saponin on bacteria resistance, the inhibitory effect on *E. coli* at 500 µg was higher than 250 and 125 and the effect demonstrated decreasing with decreased concentration of polyphenol, while with *Pseudomonas aeruginosa* increasing the inhibition by a decrease of polyphenols concentration.

Table 5. Antibacterial activity (inhibition zone) of polyphenol and saponin

	E. coli			*Pseudomonas*		
Concertation µg/ml	500	250	125	500	250	125
Polyphenols	13	12	11	10	13	20
Saponin	10	11	13	10	12	12

On the other hand, saponin showed increased inhibition of *E coli* with decreasing concentrations of saponin but the effect showed no change with two concentrations of 250 and 125 µg/ml, while the best inhibition of *Pseudomonas aeruginosa* at 500 µg/ml. According to certain research, polyphenol compounds can interact with bacterial cell walls, causing cell wall disintegration and then releasing the cellular contents. Damage to the cell wall diminishes a cell's resistance to various external effects, such as high or low osmotic pressure (46).

Conclusion

In this study, the active compounds were extracted from the *Castanea sativa* (polyphenol and saponin) and studied the effect of these extracts on LOX activity were purified from Cow's liver and *E. coli* and *pseudomonas* bacteria. These extracts showed an inhibitory effect on LOX activity and bacteria resistance which are causing more inflammatory infections in mammals' cells.

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Authors contributions

NI put the project idea and worked with HN on all procedures, also writing the article, MB review the article at the end.

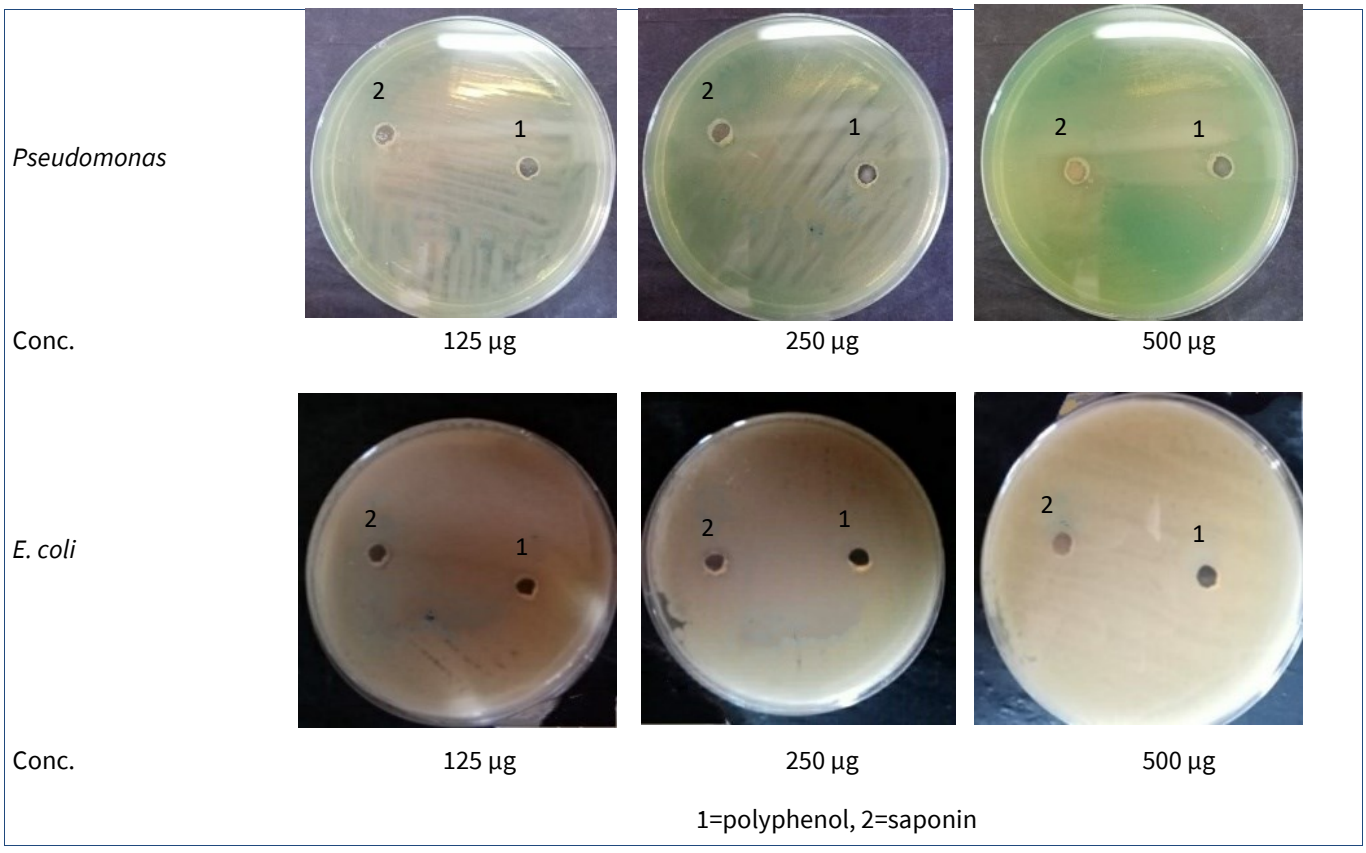

Fig. 8. Effect of the polyphenol and saponin on *E. coli* and *Pseudomonas.*

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

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