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Original article

Lycopene augments and enhances anti-oxidant/antibacterial efficiency of ethanolic leaf extract of *Helianthus annuus* over multidrug-resistant bacterial isolates

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ABSTRACT

Lycopene, the potential antioxidant naturally occurring in red carotenoid pigment is found in many fruits and vegetables. In this work, antioxidant power and antimicrobial potentials of lycopene against the multidrug-resistant (MDR) *Streptococcus agalactiae* (*S. agalactiae*) and *Streptococcus pyogenes* (*S. pyogenes*) were studied when added to ethanolic leaf extracts of *Helianthus annuus* (*H. annuus*). Supplementation of the lycopene along with ethanolic leaf extract of *H. annuus* indicates a 30 % enhancement in the antioxidant activity by 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay and reduction of 24 % reactive oxygen species (ROS) in human fibroblast cells under calcium stress compared to leaf extract alone. The antibacterial activity of the leaf extract + lycopene showed improved bacterial inhibition as low as 40 and 70 µg of the leaf extract compared with extract alone against *S. pyogenes* and *S. agalactiae* respectively. Taken together, the observations show that the natural anti-oxidant, lycopene, when added to the extract enhanced the antibacterial activity at lower concentrations which could possibly reduce the larger dose as observed in alternative or complementary medical practices.

1. Introduction

Traditional and folklore medicinal literatures (Laurieri and Delgoda, 2017; Shahat et al., 2017) have a huge reference of herbal extracts and formulations which needs to be scientifically evaluated to suit modern medical practices. In recent times antibiotic drug resistance (Medina and Pieper, 2016) has emerged as a huge global problem with increasing risk of multidrug-resistant forms evolving due to antibiotic misuse (Llor and Bjerrum, 2014), immunodeficiency (Denegre et al., 2019) and environmental mutations (Cantón, 2009). A large chuck of investment has been made in these two decades to screen small molecule libraries (Zulauf and Kirby, 2020), synthetic and semi-synthetic derivatives (Jeya et al., 2011), natural products (Leisner, 2020) and secondary metabolites (Gorlenko et al., 2020) and finally crude extract (Mohamed et al., 2020) as original traditional formulations.

The bioactivity of *H. annuus* flower and seeds is well documented in the literature (Amirul, 2020). There are studies which shows the ozonated sunflower seed oil has a wide range of antibacterial and antifungal activity (Sechi et al., 2001). There are few other studies that describe the *H. annuus* leaf extracts exhibiting antibacterial activities (Akpor et al., 2019). A notable point is sunflower plant extracts have shown antibacterial activity against both gram +ve and gram −ve organisms (Mutlu-Ingok et al., 2020). Drug resistance especially in streptococcus species has been

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well studied (Alves-Barroco et al., 2020). There are significant infections caused by S. *agalactiae* including meningitis, urinary tract infections and pneumonia while *S. pyogenes* is known to cause scarlet fever, streptococcus septic shock etc are every day threat in pediatric and geriatric populations (Al-Bayati et al., 2020). Drug resistance has become a common problem especially with these streptococcus species and the high demand for new and effective drugs (Nayak et al., 2019). Further, extracts or natural products, small molecules and secondary metabolites are known to exhibit t toxicity to the host tissues poses a bigger hurdle in the drug screening studies (Şeremet et al., 2016). Though *H. annuus* products are known to be antibacterial and their mechanism of action does not involve a pathway to describe, not much toxicity to the normal cells of the host is reported (Fatrcová-Šramková et al., 2015).

Many of the screened natural products including herbal extracts have less efficacy and bioavailability thus need a larger dose for longer periods (Bhattaram et al., 2002; Kesarwani and Gupta, 2013). This was overcome in the last few years with the new concept of combination or added naturally occurring compounds which is known to have profound bioactivity or antioxidant itself (McCarrell et al., 2008). Adding an antioxidant enhances the extracts bioactivity at lower concentrations while increasing the bioavailability and acts as a drug delivery system (Kanellos et al., 2013). Lycopene, vitamin C and other pigmented natural products are usually supplemented with drugs and food products or given as such to enhance antioxidant levels as a disease management strategy (Yonar et al., 2019). However, the idea of these antioxidants along with herbal extracts are unusual in drug screening studies.

Therefore, in the current study, evaluation of ethanolic extracts of leaves of *H. annuus* was tested for their antioxidant property and supplemented with known antioxidant lycopene to enhance oxidant scavenging as well as antibacterial activity at very low concentrations. This will pave the way for the use of safe drugs which will be only active against bacterial cells without harming the host tissues.

2. Materials and methods

2.1. H. annuus leaf extract preparation

Fresh leaves of *H. annuus* (Asteraceae), commonly known as the sunflower plant were gathered from Abha, Saudi Arabia, cleaned and shade dried. The dry leaves were pounded and kept in airtight dark containers. The ethanolic extract was prepared as per standard protocol adopted in the laboratory (Ibrahim et al., 2021). Briefly, 10 g of the grinded leaf were blended with 200 mL of absolute ethanol and agitated for 50 h. The mixture was centrifuged at 5000 rpm two times to get rid of solid materials and dried at 55 °C. A stock solution (1 %) was prepared by dissolving the dried dimethyl formamide (DMFO, Sigma-Aldrich) and sterilized utilizing 0.45 μm syringe filter.

2.2. Culture of microorganisms

Culture, maintenance, source of bacterial strains (MDR isolates; *Streptococcus pyogenes* and *Streptococcus agalactiae*) and antibiotic sensitivity assays were done as previously described by Alshahrani et al. (2022).

2.3. Disc diffusion method

A 100 μL (10^8 cfu/mL) of the logarithmic phase of test bacteria were spread on the surface of MHA plates. Ethanolic *H. annuus* extract discs were placed at a 2 cm space between discs in the MHA plates. All plates were kept at 37 °C for 48 h. The preparation showing least zone of clearance was considered and compared with the positive antibiotic controls (Bshabshe et al., 2020).

2.4. Determination of minimum inhibitory concentration

Alamar blue (AB) microplate adaptation was used to determine the minimum inhibitory concentration (MIC). Briefly, the bacterial cultures of *S. pyogenes* and *S. agalactiae* were adjusted to 4.2X 10^5 CFU/ml and 5.3 X10^5 CFU/mL equivalent to 0.002 at OD_{600} respectively. Penicillin and erythromycin served as positive controls while media served as a negative control. For MIC, one concentration before and after the complete zone of clearance was selected. Therefore, for the current experiment extract (100, 125 and 150 μg for *S. pyogenes* & 150, 175 and 200 μg for *S. agalactiae*) and extract + lycopene (40, 50 and 60 μg for *S. pyogenes* & 70, 80 and 90 μg for *S. agalactiae*) were used. The extract and test organisms were incubated along with alamar blue for 12 h in 10 replicates. The fluorescence was measured at 530 nm and 590 nm respectively at 0, 6, and 12 h. MIC was noted as the concentration at which the purple color of alamar blue is not reduced to bright red fluorescence.

2.5. DPPH assay

Antioxidant activity of the ethanolic leaf extract of *H. annuus* was determined by DPPH assay (Akar et al., 2017). Briefly, different concentrations of the leaf extract alone or with lycopene were added to 0.3 mM methanolic solution of DPPH in a ratio of 1:1. Graded doses of 5, 25, 50, 75, 100, 125, 150,175 and 200 μg/mL of extract alone or with 1 μM lycopene were used to screen the scavenging activity. The mixture was incubated for 30 min at room temperature in dark. The change in color was measured at 517 nm. DPPH solution and methanol served as positive and negative controls respectively. Graded doses (10–100 μg/ml) of L-ascorbic acid were taken as the standard reference. Percentage-free radical scavenging of the sample was calculated as follows.

$$\text{Free radicals cavenging activity}(\%) = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

2.6. Reactive oxygen species detection

Reactive oxygen species (ROS) detection Assay Kit (BioVision, Catalog # K936, CA 95,035 USA) was used as per manufacturer instructions adapted to microplate reader protocol. Briefly 2.5×10^4 human fibroblast cells were seeded in a 96-well cell culture plate, kept at 37 °C and 5 % CO_2 for 18 h to get ~ 75–85 % confluency. At the test day, cells were treated with calcium solution (100 μM in culture medium) for 30 min to create calcium stress, washed once with phosphate-buffered saline (PBS) and incubated with 100 μL/well 1X ROS Label (diluted in ROS Assay Buffer) for 45 min at 37 °C in the dark. Post incubation, the ROS label was washed and then 100 μL of blank (reagent), positive and negative control along with extract or extract + lycopene were added in triplicates for 10 min. The cells were once again washed and loaded with PBS (100 μL) and fluorescence was measured instantly at Ex/Em = 495/529 nm in end point mode. The ROS was determined as the change in the fluorescence of treated over untreated after the background subtraction as instructed in the kit instructions.

Table 1
Pattern of clinical isolates antibiotic susceptibility.

Clinical Isolates Antibiotics	*S. agalactiae*	*S. pyogenes*
Ampicillin	R	R
Amoxiclav	R	R
Amikacin	NA	NA
Ceftazidime	NA	NA
Cefotaxime	S	S
Ciprofloxacin	NA	NA
Cefuroxime	S	S
Cefazolin	S	S
Gentamicin	S	S
Imipenem	NA	NA
Nalidixic acid	NA	NA
Nitrofurantoin	NA	NA
Norfloxacin	NA	NA
Erythromycin	R	S
Clindamycin	I	S
Penicillin	S	S
Rifampicin	NA	NA
Vancomycin	R	NA

NA: Not Applicable; S: Sensitive; R: Resistant; I: Intermediate.

3. Result

The antibiotic sensitivity results (Table 1) show the susceptibility pattern of *S. agalactiae* and *S. pyogenes* clinical isolates. Typically, *S. agalactiae* was intermediately susceptible to Clindamycin and showed resistance to Erythromycin and Vancomycin compared to *S. pyogenes*. Hence Erythromycin and Penicillin were selected as a positive control for *S. pyogenes* and *S. agalactiae* respectively.

The disc diffusion method outcomes (Table 2) demonstrated that ethanolic extracts of *H. annuus* were able to inhibit *S. agalactiae* at 175 µg compared to a positive control (Penicillin) (Table 3). Similarly, the extract, at 125 µg concentration, showed complete clearance zone parallel with the positive control (Erythromycin) for *S. pyogenes* (Tables 2 & 3).

Next the additive effect of 1 µM Lycopene with extracts showed (Table 2) 80 µg was able to inhibit *S. agalactiae* while 50 µg was able to inhibit *S. pyogenes* compared with their respective positive antibiotic controls (Table 3). The antibacterial effect was observed at a minimum concentration of 20 µg signifying the additive effect of Lycopene. However, the varied concentrations of the extracts and lycopene showing antibacterial activity indicated that the nature of the organism plays a major role in the susceptibility pattern to natural extracts.

Further, the ethanolic *H. annuus* extract exhibited well-marked antioxidant activity compared to Lycopene (a well-established anti-oxidant). When the combination of various concentrations of extract and 1 µM Lycopene was tested for oxidant scavenging activity, the results showed a dose-dependent incremental oxidant scavenging (Fig. 1). 100 µg showed maximum scavenging activity

Table 3
Positive controls sensitivity results as measured using the disc diffusion test.

Antibiotics	Zone of Clearance (mm)*	
	S. agalactiae	*S.pyogenes*
Penicillin	**21**	**19**
Erythromycin	**09**	**19**

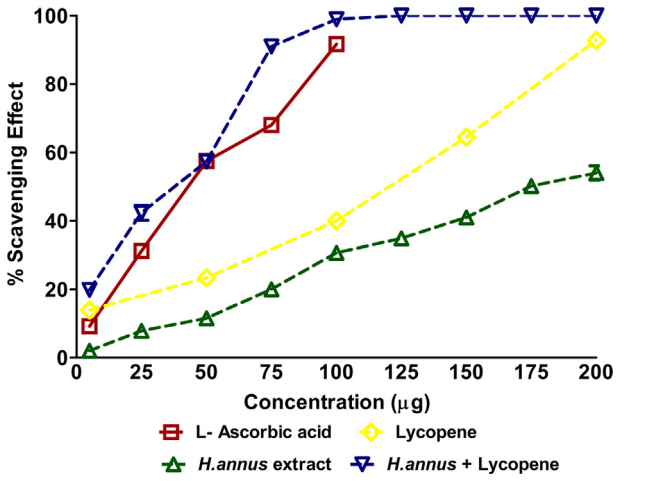

Fig. 1. The DPPH assay showing oxidant scavenging of *H. annuus* extract and *H. annuus* extract + 1 µM Lycopene.

compared with standard Ascorbic acid. In order to validate the antioxidant activity of the combined extract and Lycopene, specific ROS clearance was assessed (Fig. 2). The results well corroborated with the oxidant scavenging activity (Fig. 1) showing an accelerated ROS clearance with extract and Lycopene combination.

Finally, the MIC was determined based on the concentrations of the extracts or extracts + Lycopene obtained with both the organisms by the disc diffusion method and confirmed by alamar blue method (Table 2 & Fig. 3). Accordingly, the MIC was confirmed as 175 µg and 80 µg for *S. agalactiae* by ethanoic extract of *H. annuus* and ethanolic extract of *H. annuus* + 1 µM Lycopene respectively (Fig. 3a). Similarly, a concentration of 125 µg and 50 µg exhibited complete inhibition of *S. pyogenes* by extract and extract + Lycopene respectively.

4. Discussion

The antibacterial effect of ethanolic leaf extract of *H. annuus* was well documented from the results. Though, antibacterial (Al-Shukaili and Hossain, 2019), antifungal (Lawson et al., 2019), antioxidant and antidiabetic (Saini and Sharma, 2013) activity of

Table 2
Antimicrobial activity of *H. annuus* leaf extract alone or with Lycopene.

Herb	Nature of extract	Clinical Isolates	Concentration (µg/ml) of the extract +/- Lycopene versus Zone of Clearance (mm)*												
			10	20	30	40	50	60	70	80	90	100	125	150	175
H. annuus	Ethanolic	*S. agalactiae*	-/-	-/-	-/-	-/-	-/-	-/-	-/-	02	05	08	14	17.5	19
		S.pyogenes	-/-	-/-	-/-	-/-	-/-	4	8.4	11	14	15.5	19	20	20
	Ethanolic + 1 µM Lycopene	*S. agalactiae*	-/-	-/-	-/-	07	07	13	17	19	20	–	–	–	–
		S.pyogenes	-/-	02	08	16	19	19	20	–	–	–	–	–	–

*The results are demonstrated as the average inhibition zone (mm) obtained from four independent tests.
-/-: No inhibition zone.
-: Compete clearance.
Positive activity was calculated as per zone of clearance compared to the zone of clearance obtained with susceptible antibiotics from antibiotic susceptibility testing.

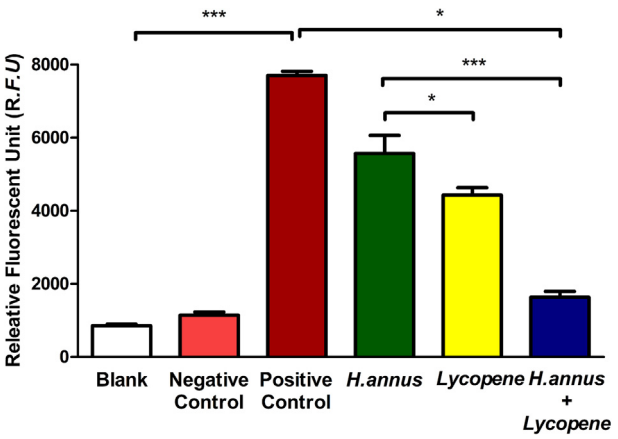

Fig. 2. ROS clearance activity of *H. annuus* extract and *H. annuus* extract + 1 μM Lycopene.

the flower and essential oil of *H. annuus* are known, traditional and flock lore uses leaves for treatment of wounds in humans and animals for long (Gai et al., 2020). Though, many plant-based formulations have shown antibacterial activities including *H. annuus* products, additive profound effects when mixed with standard natural plant based pure products are currently trending (Cowan, 1999; Safi and Al-Mariri, 2014). This is owing to extracts added with pure natural products complexity in mechanism of action that may overcome drug resistance a common phenomenon observed in recent times (Gonelimali et al., 2018). The additive effects of Lycopene along with ethanolic extract were well in agreement with other studies done similarly with different herbs and nanoparticles (Ozen et al., 2011; Zhang et al., 2017).

It is evident from literature and current results; that the herbal extracts and natural products from plant origin are effective in antibacterial activity and it has been noted that several parts of *H. annuus* and extracted with different solvents show varied of bio-activity. For instance, aqueous extracts from flowers and seeds have been implicated to be active against gram-ve organisms (Liu

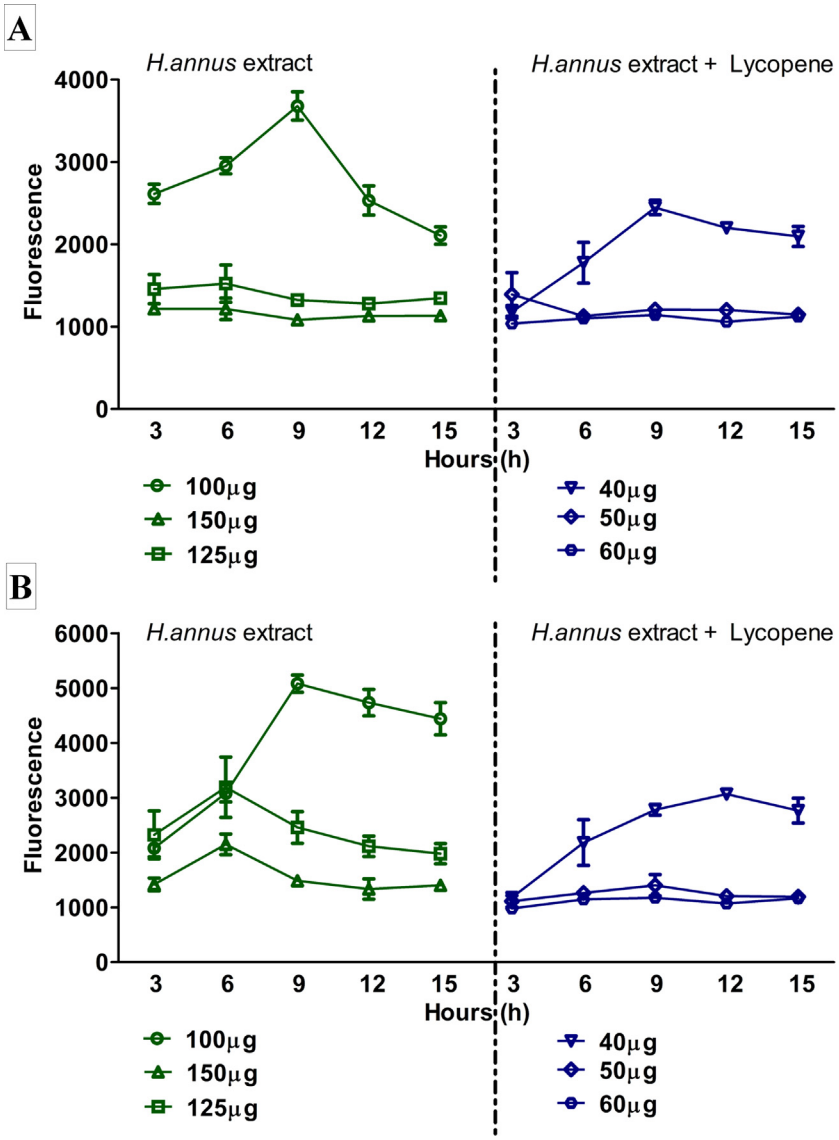

Fig. 3. A: MIC determination of *H. annuus* extract and *H. annuus* extract + 1 μM Lycopene against *S. agalactiae*. B: MIC determination of *H. annuus* extract and *H. annuus* extract + 1 μM Lycopene against *S. pyogenes*.

et al., 2020) while leaf extracts on both gram +ve and gram −ve organisms (Amirul, 2020; Sechi et al., 2001). *H. annuus* extract and Lycopene are known for its antioxidant activity, many studies have suggested its antibacterial activity to be directly killing the cells. The difference between the activities exhibited for gram +ve or gram −ve are due to its affinities towards membrane proteins on the organism itself (Mutlu-Ingok et al., 2020). However, the results of the current study showed dose-dependent clearance which might explain the inhibition of bacterial cells by direct killing.

The enhanced MIC of the extracts with the addition of Lycopene at a lower concentration is synonymous with other studies where other antioxidants like vitamin C which is naturally present or added (38). This offers a new set of natural product formulations that could very well overcome the problem of drug resistance and the question of the in-vivo toxicity observed with chemical substitutes, and small molecules (Khameneh et al., 2019; Silver, 2011). Therefore, it may be assumed that the traditional formulations like ethanolic extracts of herbal leaves along with additives like naturally occurring antioxidants may boost antibacterial activities or other bioactivities.

5. Conclusion

The ethanolic extracts of *H. annuus* along with 1 μM Lycopene a known antioxidant over its profound antibacterial activity at lower concentrations interestingly seems to be a new formulation. Further, the antioxidant nature of the sunflower and its essential oils are known, however, added Lycopene to the leaf extract not only elevates its antioxidant potential but also antibacterial effects. This will in turn reduce the large dosage of herbal formulation usually observed in traditional medical practices.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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