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IMMUNOMODULATING, ANTI-BACTERIAL AND ANTI-CANCER POTENTIAL OF ZA'ATAR (*THYMUS VULGARIS*) AND ITS COMBINATION WITH ESSENTIAL OIL (OLIVE AND BALSAM OIL)

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ABSTRACT: Introduction: *Thymus vulgaris* (za'atar) is one of the most famous and traditional spices of Arab countries. Extracts of *Thymus vulgaris* (zatar) found to be useful as traditional medicine. The current studies are aiming to further explore the phytoconstituents of *Thymus vulgaris* and its biological activity. **Methodology:** The present study investigated the phytochemical constituent of za'atar and fractionated with organic solvents using the classical method. Biological activity was done as an immunomodulatory activity, anti-bacterial, cytotoxic and anti-cancer activity *in-vitro*. **Results:** Hundred gram of ethanolic crude extract was prepared from za'atar leaves using classical method and fractionated with organic solvents (*n*-hexane, chloroform, ethyl acetate, and *n*-butanol) yielding 20 g, 16 g, 8 g and 18 g of each fraction respectively, phytochemical studies showed positive results for alkaloids, carbohydrates/glycosides, tannins, flavonoids, and triterpenes/sterols. All fractions except butanol showed potential immunomodulatory activity. Mild, significant antibacterial activity was found in chloroform fraction. Whereas, ethyl acetate and *n*-butanol samples exhibited significant activity against *E. coli*. Our results demonstrated that ethanolic extract of za'atar possesses significant activity against *Salmonella typhi*, *Staphylococcus aureus*, and *Bacillus subtilis*. Furthermore, for anticancer potential butanol fraction was found to be slightly effective against HeLa cancer cells with percent inhibition of 25%. All the extracts were tested for cytotoxicity and found to be nontoxic to fibroblast cells. **Conclusion:** Results suggested that za'atar extracts and fractions possess significant biological activity including inhibition of free radical generated during the process of inflammation, bactericidal activity against pathogens and limited anticancer activity with no cytotoxicity. Hence, za'atar could be a lead molecule for the cure of various ailments.

INTRODUCTION: Medicinal plants have been used for thousands of years ago for treating various diseases of human beings and animals and are still in use by a great percentage of the world population for being well. Some scientists are trying to analyze the traditional medicines for their beneficiary effects, and the results are promising.

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Extracts of *Thymus vulgaris* are useful in traditional medicine because of their anti-asthmatic, bronchodilator, antiseptic, antispasmodic, antitussive, antibacterial, antifungal and antiviral activities [1, 2]. Also, these extracts have shown immunomodulating properties [3, 4].

Thymus vulgaris is also quoted by various authors for its polyphenol and flavonoid contents and its potent antioxidant and free radical scavenging, anti-inflammatory, vasorelaxant, antiplatelet, antithrombin, anti-hyperlipidemic and anti-diabetic properties [5]. Recent studies [5, 6, 7] also shows that supplementation with *T. vulgaris* as an herbal

remedy has shown the remarkable antihypertensive effect and marked an improvement on hypertension-related biochemical changes and aortic vascular damage in rats. In aromatherapy, the distinct types, thymol, 'red thyme oil,' linalol type are used for its very gentle soft action and thymol for antiviral properties. A rectified product, 'white thyme oil' is also used, and it is milder on the skin. Applied to the skin, thyme relieves bites, stings and relieves sciatica and rheumatic aches and pains. Thyme is useful for ringworm, athlete's foot, thrush, and other fungal infections, as well as scabies and lice [8].

In Morocco, Jaafari [9] published a paper where *T. vulgaris* essential oils, as well as two pure products (carvacrol and thymol), were tested for their antitumor 18 activities against P815 mastocytoma cell line using colorimetric MTT assay. While all these products showed a dose-dependent cytotoxic effect, the carvacrol (with IC_{50} less than 0.004% (v/v)) was the most cytotoxic one compared to the others. The IC_{50} of thymol was 0.015% (v/v). Interestingly, when these products were tested against the normal human peripheral blood mononuclear cells (PBMC), they (except thymol) showed a proliferative effect instead of a cytotoxic one. Thymol had a cytotoxic effect on the PBMC [10].

In China, tested the essential oil of *T. vulgaris* for its *in-vitro* toxicology against three human cancer cell lines, PC-3, A-549, and MCF-7 cancer cells. *T. vulgaris* essential oil exhibited cytotoxicity towards three human cancer cells on PC-3, A549 and MCF-7 tumor cell lines [11]. This research work is designed to investigate natural as well as a traditional way to combat immunological disorders and nasty cancer and anti-bacterial properties of nature, in a hope to get a therapeutic agent that would be free from potential side effect and must be cost effective.

MATERIALS AND METHODS:

Plant Material and Extraction: The genus *Thymus* comprising of herbs and sub-shrubs is predominantly Europe and North Africa locally known "za'atar," a member of the family Lamiaceae, which grows in several regions in the world. The plant leaves were obtained from Saudi Arabia as purified form; the plant was identified and authenticated by Dr, Naira Nayeem Ph.D., Phytochemist, Faculty of Pharmacy, Northern Border University, Rafha, Saudi Arabia. Air–dried powdered of *Thymus vulgaris* leaves (500 g) was subjected to percolation with 10-fold ethanol 70%. The combined alcoholic extracts were concentrated under reduced pressure at a temperature 40 °C till dryness to yield (70 g) of total extract. The concentrated ethanol extract was mixed with 0.5L of deionized water and partitioned several times with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol, then concentrated under reduced pressure at 40 °C to give 20 g, 16 g, 8 g and 18 g, respectively using the classical method.

Phytochemical Studies were carried out on all fractions by reported method [12]. The tests were performed to find out the presence of alkaloids (Mayer's test, Wagner's test, and Dragendorff's test), carbohydrates / glycosides (Molisch test, Fehling Test), tannins ($FeCl_3$ Test), saponins (Froth Test), flavonoids (Sodium hydroxide test), triterpenoids (Acetic anhydride test) and anthraquinones (Borntragers Test). All chemicals used were of analytical grade.

Immunomodulating Activity: Luminol-enhanced chemiluminescence assay was performed[13], various concentration of extracts hexane (A) ethanol (B) chloroform (C) ethyl acetate (D) and butanol (E) (50, 150 and 250 µg/mL) was incubated at 37 ºC for 15 min in the thermostat chamber of luminometer with whole blood in another set of experiment. After incubation intracellular reactive oxygen detecting probe luminol working solution (7×10^{-5} M), and serum opsonized zymosan (SOZ) was added into each well except blank wells (containing only $HBSS^{++}$). The oxidative burst ROS production was monitored with the luminometer for 50 min in the repeated scan mode. The level of the ROS will be recorded as total integral readings as relative light units (RLU).

Determination of Cytotoxicity and Anti-Cancer Activity: HeLa cells and 3T3 mouse fibroblast, cells were maintained at sub-confluence in DMEM medium supplemented with 10% FBS, 1% Pen/ Strep (penicillin, streptomycin), and 1% glutamine. Cell lines were maintained in a humidified incubator at 37 °C with 5% CO_2. To find cytotoxic effects of Za'atar on 3T3 (6×10^4) cells were plated in a 96-well flat bottom plates for 24 h to allow the

attachment of the cells. The media was replaced, and various concentration of the test compounds (50, 150, 250 µg/mL) was added into the wells, and the plates were further incubated for 48 h. The medium was again removed by flipping the plate. MTT solution (200 µl) was added to the wells, and the plates were incubated for 4 h at 37°C. The solutions were aspirated from each well, and the cells were mixed with 100 µl of DMSO for 15 min on a rocker to dissolve the formazan crystals.

The absorbance is measured at 570 nm using microplate Spectra Max 340 (Molecular Devices, CA, USA) and the IC_{50} values were calculated. The anticancer activity of the test compound was determined by using the same method as above the cells were replaced with HeLa cervical cancer cell line at a concentration of 5×10^5 cells/mL. Cycloheximide was used, as a positive control to induce cellular toxicity [14].

Anti-microbial Activity: Agar well diffusion method was used to investigate anti-microbial activity following reported procedure [15]. The test sample was used in a concentration of 3 mg/mL, and zones of inhibition were measured mm ± standard deviation. The activity of tested samples was studied against the *Staphylococcus aureus* (AICC25923) and *Bacillus subtilis* (NCTC8236) *Pseudomonas aeruginosa* (AICC27853), *E. coli* (AICC25922) and *Salmonella typhi* (ATCC19430).

Data Analysis: The results were expressed as a percentage and mean ± standard deviation (S.D.). P-values less than 0.05 and 0.005 were considered statistically significant *P< 0.05** P< 0.005.

RESULTS: The initial phytochemical screening of ethanol, hexane, ethyl acetate, chloroform and *n*-butanol fractions of *Thymus vulgaris* extract revealed that all fractions contain alkaloids, carbohydrates, tannins, and triterpenes/sterols while anthraquinones and saponins were not found in any fraction **Table 1**. The constituents such as alkaloids, carbohydrates, tannins, flavonoids, anthraquinones have curative activity against various ailments including certain pathogenic organisms which justifies its use as traditional medicine.

TABLE 1: SCREENING OF PHYTO-CONSTITUENTS IN *THYMUS VULGARIS*

Phyto-constituents	Present/absent
Alkaloids	Present
Carbohydrates/glycosides	Present
Tannins	Present
Saponins	Absent
Flavonoids	Present
Triterpenes/sterols	Present
Anthraquinones	Absent

Effect of *Thymus vulgaris* as Anti-bacterial Agent: Anti-microbial activity of all four, *i.e.* hexane (A) ethanol (B) chloroform (C) Ethyl acetate (D) fraction was carried out against some gram-positive and gram negative bacterial strains mentioned in **Table 2**. All fractions were found to be significantly effective against *Staphylococcus aureus* with percent inhibition of 58 ± 0.8%, 64 ± 0.6%, 75 ± 0.5% and 68 ± 0.55% respectively as well as against *Bacillus subtilis* 62 ± 0.2%, 63 ± 0.2%, 76 ± 0.6% and 65 ± 0.4% respectively. Inhibition of gram-positive bacteria suggesting that may be the compound has an inhibitory effect on cell wall peptidoglycan.

TABLE 2: ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS AGAINST SELECTED MICROORGANISMS

	% inhibition ± SDV at 200 µg/mL				
Bacterial strains	**Hexane (A)**	**Ethanol (B)**	**Chloroform (C)**	**Ethyl acetate (D)**	**Standard (Ofloxacin)**
Gram +ve bacterial strains					
Staphylococcus aureus (ATCC25923)	58 ± 0.8*	64 ± 0.6*	75 ± 0.5**	68 ± 0.5*	94.7
Bacillus subtilis (NCTC8236)	62 ± 0.2*	63 ± 0.2*	76 ± 0.6**	65 ± 0.4*	92.5
Gram -ve bacterial strains					
Pseudomonas aeruginosa (ATCC27853)	16.5 ± 0.1	22 ± 0.3	12 ± 0.2	18 ± 0.1	92.4
Escherichia coli (ATCC25922)	13 ± 0.2	20.5 ± 0.1	ND	7 ± 0.08	88
Salmonella typhi	35 ± 0.2	61.5 ± 0.2*	0.8 ± 0.01	17 ± 0.2	94.1

ND= not determined *p<0.05**p<0.005

On the other hand, the gram-negative bacteria remain unaffected by the compound and its fraction because of the presence of lipopolysaccharide on the outer surface that doesn't allow these fractions to enter in the bacterial cell. Except the ethanolic extract that significantly showed an inhibitory effect against *Salmonella typhi* that is gram negative pathogenic bacteria with percentage

inhibition of 61.5% p<0.05. Ofloxacin was used as a standard drug that showed % inhibition against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *E. coli*, and *Salmonella typhi* as 94.7%, 92.5%, 92.4%, 88%, and 94.1% respectively. Overall, hexane (A) ethanol (B) chloroform (C) ethyl acetate (D) fraction showed significant activity against gram-positive bacteria P-value <0.05 and <0.005 shown in **Table 2**. Whereas only *Salmonella typhi* as gram-negative bacteria inhibited significantly by ethanol fraction and moderately by hexane fraction of za'atar. None of the fraction has any biological activity against *E. coli* and *Pseudomonas aeruginosa*.

Effect of *T. vulgaris* on Immunomodulation: Immunomodulatory properties of crude extracts and fractions were evaluated using oxidative burst assay by chemiluminescence. The whole peripheral blood was activated with zymosan and incubated with compounds results showed significant inhibitory activity P<0.05 against reactive oxygen species (ROS) production using 3 concentrations of compounds 250, 50, 5 µg/mL. following compound showed significant immuno-modulatory activity hexane (A) ethanol (B), chloroform(C), ethyl acetate (D) with an IC_{50} value of 38.7 ± 6.5, 42.0 ± 11, 30.2 ± 4.5, 31.3 ± 2.3 respectively **Table 3**.

TABLE 3: IMMUNOMODULATORY ACTIVITY OF PLANT EXTRACTS USING CHEMILUMINESCENCE TECHNIQUE ON ACTIVATED WHOLE BLOOD PHAGOCYTES

S. no.	Sample 250 µg/mL	Average IC_{50} ± SDV µg/mL
1	Hexane (A)	38.7 ± 6.5*
2	Ethanol (B)	42.0 ± 11*
3	Chloroform (C)	30.2 ± 4.5*
4	Ethyl acetate (D)	31.3 ± 2.3*
5	Butanol (E)	189.1 ± 23.8
6	Ethanolic + olive oil (1:1)	152.0 ± 17.8
7	Ethanolic + Balsam oil (1:1)	>250 µg/ml
8	Ibuprofen	43.4 ± 0.9

*P<0.05

Effect of *Thymus vulgaris* on Cell Viability and Anti-cancer Activity: Effect of za'atar and its extracts on cell viability was determined on 3T3 fibroblast cells, the percentage of inhibition was determined and compared with the standard drug Cyclohexamide. The data is presented in **Table 4**. The extract was added at final concentration of 30 µg/mL, and cells were incubated for 48 h. The result showed no significant inhibition for all the given extract and fractions, the percent inhibition is found to be below 15%. However, the butanol extract of za'atar found to be slightly toxic on fibroblast cell with 25% inhibition of cell growth. These compounds appear to be far less toxic when compared to the cycloheximide, that is inhibiting the cell growth up to 71%.

TABLE 4: CYTOTOXIC ACTIVITY OF PLANT EXTRACTS USING MTT ASSAY ON 3T3 FIBROBLAST CELLS AND CERVICAL CANCER CELL LINE AT FINAL CONCENTRATION OF 30 µg/ml

S. no.	Sample 30 µg/mL	Average % inhibition HeLa cervical cancer cells	Average % inhibition 3T3 fibroblast cells
1	Hexane (A)	2.0 ± 0.04	23 ± 0.04
2	Ethanol (B)	1.0 ± 0.03	2 ± 0.02
3	Chloroform (C)	3.0 ± 0.06	1 ± 0.02
4	Ethyl acetate (D)	20.3 ± 0.4	12 ± 0.1
5	Butanol (E)	2.0 ± 0.01	25 ± 0.7
6	Ethanolic + olive oil (1:1)	2.1 ± 0.06	5 ± 0.04
7	Ethanolic +Balsam oil (1:1)	2.1 ± 0.04	15 ± 0.08
8	Doxorubicin	71.3 ± 4.0	-
9	Cyclohexamide	-	71.4 ± 0.05

Thymus vulgaris extracts and fractions were analyzed for its anti-cancer activity on HeLa cervical cancer cell line. The data presented in **Table 4** indicated that *Thymus vulgaris* had not inhibited the cancer cell viability significantly except extract of ethyl acetate marginally inhibited the cancer cell growth with percent inhibition of 20% and results were compared with standard drug doxorubicin that is inhibiting the HeLa cells with 71.3% at the same concentration.

DISCUSSION: The results of our investigation confirmed the rationale for the medicinal use of the *Thymus vulgaris*. It is a famous herb of Arab, and they traditionally consume it with olive oil as a regular food. Our study based on biological effects of *Thymus vulgaris* alone as well as its synergistic effect with olive oil and balsam oil in a 1:1 ratio. The current study has revealed significant biological activities as an antibacterial, anti-cancer and immunomodulatory effect of za'atar.

The aerial part of the plant was used to perform biological and phytochemical studies; in addition to that, the fractions were also prepared using different solvent systems including chloroform, ethyl acetate *n*-hexane, and butanol. Phytochemical studies showed that active ingredients of the plant are flavonoids, triterpenes, sterols, tannin, anthraquinone, alkaloid also reported in one study that flavonoid is major component of *Thymus vulgaris* [16]. Chemical constituents in the plants or crude extracts are known to be biologically active ingredients, and some other active chemical constituents are considered as secondary metabolites components. They are directly responsible for different activity such as antioxidant, antimicrobial, antifungal and anti-cancer.

Other studies also confirmed the presence of the flavonoids, saponins and steroids compound in all crude extracts but alkaloids, triterpenoids, and tannins are not essentially present in all crude extracts [17]. Za'atar when screened against pathogenic bacterial cultures it was found mild, significant antibacterial activity in chloroform fraction. Whereas, ethyl acetate and *n*-butanol samples exhibited remarkable activity against *E. coli*. *E. coli* is one of the very well understood pathogens leading to some diseases including diarrhea, enteric hemorrhage, and typical multi-drug resistant urinary tract infection [18, 19].

Our results demonstrated that ethanolic extract of za'atar exhibited significant activity against *Salmonella typhi*, *S. aureus,* and *Bacillus subtilis*. Among them, *S. typhi* and *S. aureus* are pathogen of great concern since their infection may be related to improper hygiene so consuming thyme as regular diet would give an option to become immune against such infections. Other studies also revealed previously that essential oil from *Thymus vulgaris* and its synergistic effect with another plant extract *Pimpinella anisum* possess antimicrobial activities against pathogenic micro-organisms [20, 21]. An earlier study has uncovered the fact of thyme essential oil that is toxic to consume orally [22]. Similarly, another plant *Mentha pulegium* of same Lamiaceae family is used to make herbal teas, which, although not proven to be dangerous to healthy adults in small doses, is not recommended, due to its known toxicity to the liver [23].

Consumption of isolated essential oil from these plants can be fatal to infants and children; however, our study has shown that *Thymus vulgaris* extracts, its fractions and as well as synergistic combination with olive and balsam oil showed no toxicity *in-vitro* against fibroblast cell line up to 30 µg/mL concentration. It means it is a much safer and least toxic herb to consume. The anticancer potential for za'atar was also evaluated on HeLa cervical cancer cell line; it was found that only butanol extract exhibited mild activity at percent inhibition of 25%.

For immunomodulating or anti-inflammatory activity *Thymus vulgaris* proved to be potential immunomodulating agents against activated whole blood phagocytic cells. Our study has shown that chloroform extract is significantly active against free radicals generated during the innate immune response might be because of its antioxidative action. A previous study by AmirGhofran Z [24] has reported the water extract of *Thymus vulgaris* as an immunomodulating agent against dendritic cells and T cells. However, we have challenged the innate immune modulation by alcoholic extract as well as different fractions of *Thymus valgaris*, and it was found to be effectively suppressed by hexane, ethyl acetate, and chloroform fractions, whereas its combination with olive oil and balsam oil suppress the individual effect of za'atar. So, consuming za'atar alone without olive oil and balsam oil would be more healing toward immune disorders.

CONCLUSION: Many studies have reported the biological activity of essential oils isolated from *Thymus vulgaris*, however current study has identified the biological potential of *Thymus vulgaris* ethanolic extracts and its organic fraction. Biological activity was studied alone and in combination with essential oil as the people consume traditionally. Synergistic effect of balsam oil was also investigated. The results concluded that *Thymus vulgaris* is significant immune modulator as well as a potential antibacterial agent. With no *in-vitro* toxicity proven by present analysis either on cancer cells and normal cells. These investigations give the insight about the potential of this herb that could be utilized for a therapeutic purpose to cure either bacterial infections particularly gram-positive or immune disorders without the fear of cellular toxicity.

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