

## ***In vitro* Effects of Some Bee Products on *T. gondii* Tachyzoites**

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**Abstract:** *Toxoplasma gondii*, is ideal opportunistic zoonotic protozoan, exceeds neurological and congenital impact sequence to flare up of latent toxoplasmosis. It is intracellular pathogen and is so difficult to treat, it requires higher doses of intracellular antimicrobial drugs which reflect repulsive side actions. So, the aim of a current study is focused to evaluate the effect of some bee products (propolis, honey and bee venom) with special emphasis to sulfa-dugs as anti-*Toxoplasma* activity. The results revealed that all investigated bee products showed destructive on viable tachyzoites which promoted directly with the different dilutions of the bee products, in particular; 100, 50, 25 and 10 respectively, at chilling temperature (4°C) and corresponding to LD<sub>50</sub> & LD<sub>100</sub>. Bee venom proofed the faster and powerful *in vitro* anti-tachyzoites action with all dilutions, exceeding even the control sulfonamides. 5, 6, 7 and 8 respectively while diluted propolis were effective to enhance the persisting viable tachyzoites course. Where 7% dilution of propolis symbolizes the maximum optimal value, successfully increases the average percent of LD<sub>50</sub> & LD<sub>100</sub> viable tachyzoites longer time a live up to 172.9 hours post exposure to propolis, exceeding about three days over the normal control saline solution. In conclusion: the results revealed that bee products are promising for development of potent and novel anti-toxoplasma drug.

**Key words:** *In vitro* • *T. gondii* Tachyzoites • Propolis • Bee Honey and Bee Venom

### **INTRODUCTION**

*Toxoplasmosis* is typical zoonoses, may be acute via tachyzoites or chronic through bradyzoites stages [1]. Acute tachyzoites stage is exceeding opportunistic human impact sequence to cerebral [2], congenital [3] and ocular toxoplasmosis [4 5] and ranks among the 10 most opportunistic and the forth deadly pathogen in immune-suppressed patients [6].

Apitherapy or the medical uses of bee products are ranged from use as honey [7- 10], pollen [11], royal jelly [12], wax [13], propolis [10, 14] and venom [15, 16].

Treatment of acute human toxoplasmosis is so difficult, where higher doses of intracellular antibiotics or sulfonamides reveal terrible side actions [17]. Thus the aim of the present study is to evaluate bee products; honey, propolis and bee venom against *T. gondii* tachyzoites stage as safe natural materials with reference to the chemical drug sulfonamides.

### **MATERIALS AND METHODS**

***Toxoplasma gondii* Strain:** In the present study the RH strain was maintained and secured in Zoonotic Diseases Department, National Research Center, Egypt, via using regular mice peritoneal passage every 3 days for continuous collection of fresh tachyzoites [18].

**Animals:** Forty mice were obtained from Laboratory Animals House, National Research Center, Egypt. These animals were used as long as the term of the study, housed in standard environmental conditions at temperature (24°C) and relative humidity (50%) with a 12:12 light: dark cycle. with free access to a standard commercial diet and water. All animals were used for harvesting and secure the peritoneal RH tachyzoites with continuous regular flow, by successive intraperitoneally tachyzoites-tachyzoites mice cycle every 72 hours. Experiments were performed according to the Guide for

the Care and Use of Laboratory Animals and Ethical Approval of animal rights according to Committee, National Research Centre, Egypt under registration number 1-2 /0-2-1.2012. The obtained tachyzoites from mice ascetic fluid were diluted for adjusting the tachyzoites count at  $10^3$  /ml and exposed to varied experimented bee products and the control ones.

### Collection and Preparation of the Experimented Materials:

- Propolis was collected from apiary farm near El-Mansoura City, Dakahlia Province, Egypt. Ten grams propolis sample was cut into small pieces and extracted at room temperature with 50 ml of 70% ethanol (twice after 24 hours) according to Hegazi *et al.*, [19]. The extracted of propolis was 0.1 gm/1gm dissolved in 10 ml normal saline.
- The dried bee venom was obtained by the electric device unit (Apitronic Services, Richmond, B.C. and Canada). One hundred mg of dried whole bee venom was dissolved in 1 ml sterile normal saline, centrifuged at 1500 rpm for 10 mints and supernatant was collected [16].
- Coriander honey was collected from market of Egyptian origin. Five grams of honey were diluted [20] in sterile distilled water.
- Sulfonamides drug; Sulfadiazine and pyrimethamine; were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). A mixture of sulfadiazine and pyrimethamine were used in 100 ml as [21, 22].

All experimented materials were diluted as the following dilution 100, 50, 25, 10, 9, 8, 7, 6, 5, 4, 3, 2 and 1% was done.

**Estimation of Viable Tachyzoites:** Under strict sterile condition the used peritoneal harvested tachyzoites samples were counted and adjusted to  $10^3$  tachyzoites / ml of normal saline using haemocytometer. Number of tested samples were used in serial dilutions of each product (honey, propolis, bee venom and the mixture of sulfonamides) at 4°C throughout the experiment. After good shaking, different dilutions from treated tachyzoites were microscopically counted in average 10 ml by haemocytometer every 12 hours post treatment (HPT), all samples are re-examined after 8 to 6 HPT or even lower.

**Statistical Analysis:** The results obtained in the present work are represented as means  $\pm$  standard error and were analyzed using analysis of variance (ANOVA). The significance of difference between means at  $P < 0.05$  was calculated using the Duncan Multiple Range Test [23].

## RESULTS

All investigated bee products showed *in vitro* significant anti-*T. gondii* tachyzoites activity compared with the reference sulfonamides (Table 1). The results validate that the destructive power on viable tachyzoites was promoted directly with the different dilutions of any of the three bee products, particularly on 100, 50, 25 and 10 nm, at chilling temperature (4°C) and reflected by LD<sub>50</sub> & LD<sub>100</sub>. Bee venom proofed faster and powerful *in vitro* anti-tachyzoites action with all dilutions (Table 1) than the other bee products exceeding even the control sulfonamides (Table 1). 5, 6, 7 and 8% diluted propolis was effective to enhance the persisting viable tachyzoites course (Table1). Where 7% of propolis symbolizes the optimal value, successfully increases the average of LD<sub>50</sub>

Table 1: The LD<sub>50</sub> & LD<sub>100</sub> percentages of different tested agents against *T. gondii* tachyzoites

Agent exposed to $10^{(3)}$ tachyzoites/ml	Honey				Bee venom				Propolis				Mixture of Sulfadiazine and pyrimethamine			
	Persistence time & % of LD <sub>50</sub> /HPE		End point & % of LD <sub>100</sub> /HPE		Persistence time & % of LD <sub>50</sub> /HPE		End point & % of LD <sub>100</sub> /HPE		Persistence time & % of LD <sub>50</sub> /HPE		End point & % of LD <sub>100</sub> /HPE		Persistence time & % of LD <sub>50</sub> /HPE		End point & % of LD <sub>100</sub> /HPE	
	HPE	LD <sub>50</sub> %	HPE	LD <sub>100</sub> %	HPE	LD <sub>50</sub> %	HPE	LD <sub>100</sub> %	HPE	LD <sub>50</sub> %	HPE	LD <sub>100</sub> %	HPE	LD <sub>50</sub> %	HPE	LD <sub>100</sub> %
Dilution																
100%	1/2	0.45	1	0.9	1/2	0.45	1/2	0.4	2	1.8	4	2.9	1	0.9	4	2.9
50%	2	1.8	3	2.2	1/2	0.45	1/2	0.4	6	5.5	9	6.4	1.5	1.4	6	4.3
25%	4	3.6	7	5	1/2	0.45	1/2	0.4	12	10.9	24	17.1	3	2.7	9	6.4
10%	12	10.9	24	17.1	1/2	0.45	1/2	0.4	72	65.5	48	34.3	9	8.2	12	8.6
9%	18	16.4	48	34.3	1	0.9	1	0.7	96	87.3	108	77.1	10	9.1	14	10
8%	18	16.4	72	51.4	1	0.9	1	0.7	156	141.8	168	120	16	14.5	20	14.3
7%	48	43.6	96	68.6	1	0.9	1	0.7	192	174.5	240	171.4	18	16.4	22	15.7
6%	48	43.6	108	77.1	1	0.9	1	0.7	180	163.6	192	137.1	30	27.3	45	32.1
5%	72	65.5	108	77.1	1	0.9	1	0.7	168	152.7	204	145.7	36	32.7	58	41.4
1%	120	109	132	94.3	1.5	1.4	2	1.4	120	109	144	102.9	48	43.6	60	42.9
CS	110	100	140	100	110	100	140	100	110	100	140	100	110	100	140	100

10<sup>(3)</sup> RH strain tachyzoites, HPE : hours post exposure, LD<sub>50</sub> : lethal dose at which 50% of tachyzoites degenerated/ HPE, LD<sub>100</sub> : lethal dose at which 100% of tachyzoites degenerated/ HPE, HPE : hours post exposure CS : Control saline

Table 2: The Persistence time of *T. gondii* tachyzoites viability of different tested agents.

Dilutions	% of /HPE with Propolis	% of /HPE with bee honey	% of /HPE with bee venom	% of HPE with Sulfa-drugs
100%	2.4	0.68	0.43	1.9
50%	5.9	2	0.43	2.9
25%	14	4.3	0.43	4.6
10%	49.9	14	0.43	8.4
9%	82.2	25.4	0.8	9.6
8%	130.9	33.9	0.8	14.4
7%	172.9	56.1	0.8	16.1
6%	150.4	60.4	0.8	29.7
5%	149.2	71.3	0.8	37.1
1%	105.9	101.7	1.4	43.3
CS	100	100	100	100

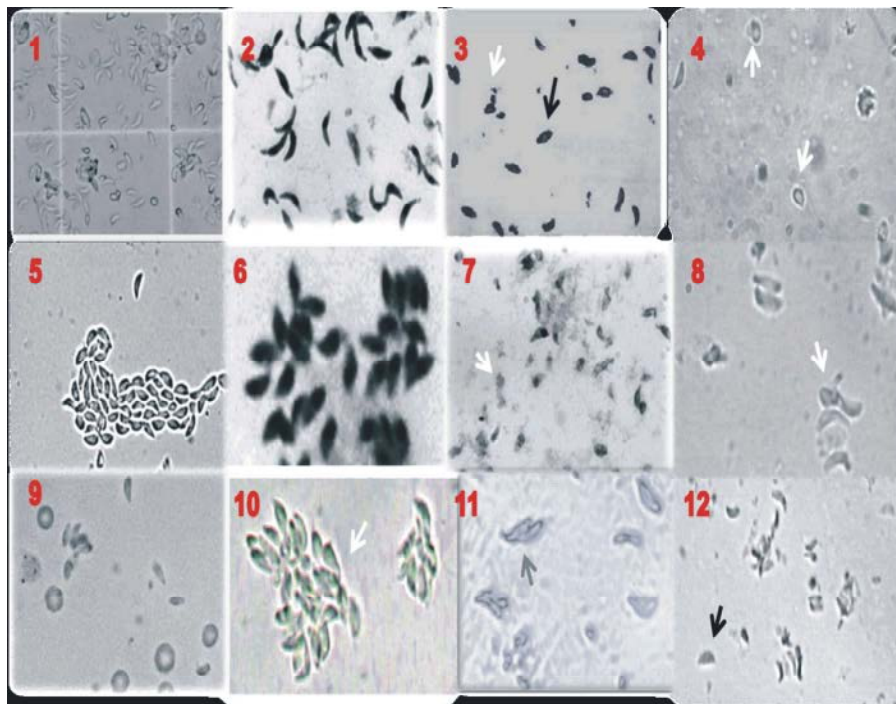


Fig. 1: Comparative light microscopy of differently treated tachyzoites: 1. Propolis, 2. Honey and 3. Bee venom.

Slid-1: Free unstained *T. gondii* tachyzoites of RH strain, were counted by using haemocytometer for adjusted and estimated both inoculated  $10^{(3)}$  and  $LD_{50}$  (X400).

Slid-2: Free unstained  $10^{(3)}$  tachyzoites, were counted and chilled in normal saline (X600).

Slid-3&4: unstained partially affected (black arrow) and completely affected (white arrow) at bee honey dilution of (50%) at  $LD_{50}$  &  $LD_{100}$  respectively (X400).

Slid-5:  $LD_{50}$  of viable tachyzoites aggregation of Propolis dilution at (7%) (X400).

Slid-6: showing swallow deformity with higher water content inside the viable tachyzoites, start of destructive phase of  $LD_{50}$  Propolis dilution at (50%) (X1000).

Slid-7: degeneration of the half number of tachyzoites ( $LD_{50}$ ), showing complete degeneration (white arrow), where, the other half number of zoites still sustained viable at propolis dilution of (10%) (X400)..

Slid-8: showing complete tachyzoites degeneration (white arrow), represent the value of ( $LD_{100}$ ) at propolis dilution of (1%) (X400).

Slid-9: Free unstained tachyzoites with RBCs, moments just exposed to bee venom dilution of 100% at (0.5/HPE) (X400).

Slid-10, 11&12:  $LD_{50}$  of moderate, highly and complete degenerative tachyzoites at bee venom dilution of (50%), (25%) & (10%) (X400).

& LD<sub>100</sub> viable tachyzoites longer time up to 172.9 HPE, exceeding about three days over the normal control saline solution at the same chilling temperature (4°C). Control sulfonamides and Honey (Table 1) were showed also a significant *in vitro* anti-protozoan activity (Fig. 1-3).

Viable tachyzoites showed (Fig. 1-2) showed semi-parallel values of both LD<sub>50</sub>&LD<sub>100</sub> with all trials as long as the term of study, with the highest effective enhancing and preserving tachyzoites values were at; 5, 6, 7 and 8% of diluted propolis, with maximum values at 7% up to 174.5 HPE. While the degenerative tachyzoites recording faster time with 100, 50, 25 and 10% of the diluted three bee products with maximum destructive values at 100%.

## DISCUSSION

The current results validate physical and chemical factors encourage *in vitro* anti-protozoal activities of honey at chilling temperature (4°C). The sugar supersaturated nature with the minute water molecules, exploit the osmotic, electrical and hygroscopic action, consequently reduce water content in tachyzoite cytoplasm with subsequence tachyzoites morphological alter [24]. Also, the acidity of honey (pH 3.2 and 4.5) is a concern factor, being unsuitable for tachyzoites viability, which sustained in normal saline [25]. Also, honey contains up to 20 kinds of both organic and aminoacids; proline one of the twenty DNA-encoded amino acids and gluconic acid, plus minor additional organic acids, consisting of formic, acetic, butyric, citric, lactic, malic acids, all possible have destructive action on tachyzoite cell membrane [26]. Gluconic acid forms the gluconate ion; quinine gluconate is a salt between gluconic acid and quinine, which is used for intramuscular injection in the treatment of malaria (apicomplexal protozoa as *Toxoplasma*) [27]. But, the anti-protozoal action of honey possibly minimized at the practical chilling temperature (4°C); where, honey turn into a supercooled liquid, in which the glucose will forms a semisolid solution of precipitated glucose crystals [24], being unexposed to suspended tachyzoites.

In the present study; bee venom proofed *in vitro* the sooner and potent anti-protozoal biogenic agent, exceeding even the control sulfonamides. This possibly referred to mellitin and phospholipase A<sub>2</sub> in bee venom are potent toxic component could be induce blood hemolysis, hemoconcentration and hyperkalemia [28]. Melittin also increases the permeability of cell membranes to ions, particularly Na<sup>+</sup> and indirectly Ca<sup>2+</sup>, resulting for a marked morphological and functional change in exposed

tachyzoites [29]. Bee venom, is holding hyaluronidase enzyme, which is often produced by bacteria as a virulence factor to destroy the polysaccharide that holds cells together, making easier for the pathogen invasion through the host tissues. Hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tachyzoites permeability [29-31]. Therefore, bee venom is possibly used as synergetic with other bee products to facilitate cell diffusion and dispersion. So, in the present study hyaluronidase enzyme possible share other toxic component for *in vitro* tachyzoites deterioration, in addition, it was observed that bee venom exposed tachyzoites set no aggregation properties even at hours prior to unaffected samples, this firmly submitted to hyaluronidase enzyme action, fast denaturants cell wall. Also, act as factor minimizing production of NO in macrophages, astrocytes and microglia cells by lipopolysaccharides [31, 32], with high suspension of possible *in vivo* superior action, promising to study further trials. Confirming the obtained results, mellitin and phospholipase A<sub>2</sub> exhibit strong anti-microbial inhibitory effects on *Borrelia burgdorferi* [32, 33]. Also, melitin can poke holes in the protective envelope which surrounds viruses, also kill the *Candida albicans* [33] and suppress *Mycoplasma hominis* and *Chlamydia trachomatis* infections [34-36]. Phospholipids A<sub>2</sub> and lysophospholipase enzymes are capable to hydrolyzing all phospholipids extractable from yeast cells [37-41]. Also Hegazi *et al.*, [16] found that whole bee venom and whole bee extract exhibited potential antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* bacterial strains, the results confirm BV useful complementary antimicrobial agent against pathogenic bacteria. These findings explain the potent action of bee venom on *T. gondii* tachyzoites.

The results of propolis in the present study were validate anti-protozoal activity, possibly associated with the presence of bioflavonoids and derivatives of caffeic acid plus phenolic acids, specific terpenoids and prenylated derivatives [19, 42-48]. Also, the results were agree with *in vitro* studies; include antibacterial [49, 50], antifungal [42, 51], antiviral [9, 52] and anti-cancer [53-56]. Results demonstrate that ethanol extract of propolis possesses clear *in vitro* anti-trichomonas activity in macrophages. Similarly mice peritoneal wash of *T.gondii* tachyzoites which was used in this experiment may contain macrophages harboring tachyzoites. MonzoteFidalgo *et al.* [57] confirm propolis extract as a natural source to obtain new antiprotozoal agents, against

both intracellular *Leishmania amazonensis* and extracellular *Trichomonas vaginalis*. Also, recording activity against protozoan causing trichomoniasis, toxoplasmosis and lamblasis [57, 58]. Hegazi *et al.* [19] observed the inhibitory effect of propolis extract on *Fasciola gigantica* eggs shell as well as Fasciola worm [48]. Also G rzia Maria de Carvalho MachadoI [59] proofed the leishmanicidal action of phenolic compounds, flavonoids and amyrins..

In the current study, it was confirmed that the addition of 7% of propolis on the preservative chilling saline, maintain viable tachyzoites three to four days over the normal time. This action may be related to propolis nourishment elements; holding protein, vitamins and minerals plus to minute glucose level [60, 61]. These molecules possibly act similar to calf serum as feeding supplement to sustained cryo-preserved tachyzoites, in addition to its antibacterial [42] and antifungal action [62]. They may also be responsible for longer viable persisting time of tachyzoites and indicated that whey higher diluted propolis (7%) was found to be effective preservative than the higher concentrations,. this is probably due to good dissolving elements, which become more directly contact with free suspended tachyzoites as flavonoids, or some of propolis active principles, which require more higher dilution with variable PH to be vigorous efficient and dynamic at chilling temperature (4 C). Also, the *in vitro* precipitating factor may be directly elevated with higher propolis concentrations at (4 C), possibly trapping down some of heavy elements which effectively could destruct tachyzoites. Accordingly the 7% diluted Propolis extract can be adapted to use in liquid nitrogen cryopreservation of *T.gondii* RH strain tachyzoites, which signify medical and economic impact, used directly viable in research purposes and for accurate golden Methylene Blue Dye Test (DT) for confirming humans and animals toxoplasmosis. Also, 7% diluted propolis extract could be used in the preservation of RH strain tachyzoites antigen used for manufacturing different types of serological kits.

From the over mentioned results it could be concluded that; bee products (Propolis, venom and honey) could be considered as potent and novel *in vitro* anti-protozoan agents against *T. gondii* acute tachyzoites stage. Thus, the knowhow application of honey products should be selectively examined separately or in mixture dosages to maximize their efficacy in further *in vivo* trials.

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