Middle Black Sea Journal of Health Science / Mid Blac Sea J Health Sci

May 2023; 9(2): 312-324

RESEARCH ARTICLE

DOI: 10.19127/mbsjohs.1253511

Amoebicidal and Cytotoxic Activity of Propolis collected from Different Regions in Turkey on *Acanthamoeba castellanii* Trophozoites

Zeynep Kolören[1(ID)], Ömer Ertürk[1(ID)], Zülal Atli Şekeroğlu[1(ID)], Ülkü Karaman[2(ID)]

[1]Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Ordu University, Ordu, Turkey
[2]Department of Medical Parasitology, Faculty of Medicine, Ordu University, Ordu, Turkey

Received: 20 February 2023, Accepted: 03 May 2023, Published online: 31.05.2023

Abstract

Objective: The present study aimed to investigate the amoebicidal effects of propolis collected from different regions in Turkey on *Acanthamoeba* trophozoites.

Method: The propolis was collected from different geographic sites (Van, Erzurum, Gümüşhane, Ordu, Rize, and Muğla) in Turkey. Different concentrations of propolis ethanolic extract (in quantities from 1, 2, 3, 4, 5, 6, 7, and 8 mg/mL) and the same volume of trophozoites in 100 µl culture were blended for the identification of the amoebicidal efficiency of propolis.

Results: The growth of trophozoites stopped in Turkish propolis extracts with 50% inhibitory concentrations (IC50)/48h for 5 mg/mL extract solution. Propolis showed more potent inhibitory effects on *Acanthamoeba* trophozoites at concentrations of 7, 6, 5, 4, and 3 mg/mL for 72 h. Propolis extract substantially inhibited human bronchial epithelial cells, especially at higher concentrations (7, 8, and 16 mg/mL).

Propolis can kill *Acanthamoeba* trophozoites at a concentration (of 3-6 mg/mL) but is safe for human bronchial epithelial cells at the same concentrations after 72 h treatment, this paves the way for propolis to be an alternative source of therapeutic drugs in the treatment of *Acanthamoeba* spp.

Conclusion: *Acanthamoeba* infection still cannot be treated with drugs. In this study, propolis collected from different regions of Turkey showed amoebic and cytotoxic activities. Propolis extract, which is a natural product that can be used against *Acanthamoeba* trophozoites can be an alternative source of therapeutic drugs in the treatment of Acanthamoeba, supported by in vivo studies.

Keywords: *Acanthamoeba* sp., amoebicidal activity, Turkish propolis, MTT assay, Cytotoxicity, RP-HPLC

Suggested Citation: Koloren Z, Erturk O, Atli Şekeroglu Z, Karaman U Amoebicidal and Cytotoxic Activity of Propolis collected from Different Regions in Turkey on Acanthamoeba castellanii Trophozoites. Mid Blac Sea Journal of Health Sci, 2023;9(2): 312-324

Address for correspondence/reprints:

Zeynep Kolören

Telephone number: +90 (505) 551 69 25

E-mail: zeynep.koloren@yahoo.com

Note: This study was presented as a poster presentation at the SEAB 2017, 3rd International Symposium on Eurasian Biodiversity, (05–08 July 2017, Minsk-Belarus).

INTRODUCTION

Propolis is a mix of content used by bees to protect the hive. This preservation refers to filling gaps in the hive's walls, decreasing entry on cold days, and mummifying the claimed uninvited guests, thus avoiding their decomposition. Bees take resins from sprouts, exudates, and other parts of plants. They mix those resins with their salivary enzymes and wax and then form propolis (1).

Since propolis is rich in flavonoids, resins, vitamins, and minerals, it has been preferred in natural treatments in public health and applied to medicine for many years. It is known that propolis contains different compounds depending on the plant species and geographical conditions where it is collected (2). The chemical compound of propolis is based on the local flora of the districts and collection time, which makes it difficult to standardize (3).

Recently, many studies have been conducted on propolis because it is anti-bacterial (4); antioxidant, anticancer (5); wound healing (6), and anti-protozoal activities (7).

Propolis prevents the multiplication of promastigotes in *Leishmania*, inhibits the parasitemia in *Plasmodium* and *Trypanosoma*, prevents the growth of *T. cruzi*, inhibits the proliferation of *Blastocystis*, and reduces oocysts shedding in *Cryptosporidium* and *Giardia*. Moreover, propolis increases the specific IgM and IgG titers in toxoplasmosis. Besides these, there is limited knowledge of the mechanism with the thought of these findings, the mechanism of action of propolis is not clear in *Acanthamoeba* spp., and *Trichomonas* spp. (8).

Acanthamoeba spp. is a phase of free-living amoeba that distributes in natural water sources, seawater, and soil, it has also been isolated from various niches including, swimming pools, tap water, bottled mineral water, and even lens storage containers and lens cleaning solutions, dialysis units, dental treatment units (9,10).

Acanthamoeba culbertsoni, *Acanthamoeba castellanii*, and cause granulomatous amoebic encephalitis (GAE) and *A. rhysodes*. *Acanthamoeba* keratitis (AK) is a parasitosis caused by various *Acanthamoeba* species. Asymptomatic individuals present with severe ocular pain, inflammation, visual impairment, and annular stromal infiltration. In such cases, vision may deteriorate over time and vision loss may occur (11).

There is no eradication of *Acanthamoeba* from infection yet because treatment of acanthamoebiasis still has very significant problems related to the induction of parasite resistance and toxic side effects (10). *Acanthamoeba* infection is similar to bacteria, and both of them show high resistance to many antimicrobial agents at tolerable concentrations. Therefore, there is a need for new approaches and more effective treatment protocols for *Acanthamoeba* infections.

Today, several studies conducted in many fields to eradicate severe parasitic and bacterial infections based on bioactive compounds extracted from various natural sources (11, 12, 13, 14).

The present study aimed to investigate the amoebicidal effects of propolis collected from different regions in Turkey on *Acanthamoeba* trophozoites. In many studies, it has also been shown that propolis has anti-bacterial and anti-cancer effects. However, there is limited study

on the cytotoxicity of Turkish propolis on human bronchial cells. Therefore, this study also discusses the determination of the cytotoxic activity of propolis collected from different regions of Turkey.

METHODS

Propolis Extraction

Propolis samples collected from Ordu, Erzurum, Gümüşhane, Van, Rize, and Muğla provinces of Turkey were tested. The samples (60 g) were dissolved in 300 ml of ethanol for 72 h at room temperature in a shaking incubator. After the reaction mixture was filtered to remove insoluble components, the extract was evaporated to take out the resolvent, and the dry residue was thawed in distilled water. The supernatant was used for the propolis solution's final concentration (32 mg/mL). Seven different concentrations of propolis (1, 2,,4, 5, 6, 7, 8 mg/mL) were used for treatment with *Acanthamoeba* trophozoites.

Culture of Acanthamoeba

A. castellanii (ATCC 30010 purchased from the American Type Culture Collection) was placed on Ringer agar plates seeded with *E. coli* so that it could use gram-negative bacteria as a food source. After 3 days of incubation at 26°C, the plates were examined under the microscope to detect the presence of *Acanthamoeba* trophozoites (13). The plates were incubated at 26°C in the incubator, and 3 days later, they were microscopically examined for the presence of *Acanthamoeba* trophozoites (13). Trophozoites in the exponential growth stage (72 h) were gently collected from Ringer agar plates with the aid of a sterile cell scraper. The trophozoites were washed twice with Ringer's dilution and concentrated by centrifugation. Viable and dead trophozoites were separated from each other by Trypan blue staining and counted in a hemocytometer (13). The final concentration was prepared to 10^6 trophozoites/mL. Numbers of viable *Acanthamoeba* trophozoites were determined during the 1, 3, 6, 8, 24, 48, and 72-hour periods at 26°C.

Evaluation of the effect of propolis on Acanthamoeba

The propolis extract of 100 µl was mixed with an equal volume of amoebae culture in concentrations of 1, 2, 3, 4, 5, 6, 7, and 8 mg/mL. The viability of the parasite was checked and recorded at 1-, 3-, 6-, 8-, 24-, 48-, and 72-h intervals by using a Thoma hemocytometric chamber to represent the amoebicidal activity of the propolis extract. One hundred *A. castellanii* trophozoites were counted for each experiment. A control group was formed by adding amoeba culture to the reaction mixture without propolis extract. The results are stated as percent inhibition per control cells (considered 100%). The 50% inhibitive concentrations (IC50) were shown with logarithmic regressions. All experiments were performed in three repetitions and statistical analyses were made to determine the cell viability percentage.

Cytotoxicity of propolis extract

The cytotoxicity of propolis ethanolic extracts was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (15). BEAS-2B, human bronchial epithelial cells (ATCC, CRL-9609), were used for determining the cytotoxicity of propolis. The cells were maintained in serum-free LHC-8 medium and cultured at 37°C in a 90% humidified incubator with 5% CO_2. After BEAS-2B cells were seeded in a 96-well plate ($1x10^4$ cells each well), the cells were treated with

different concentrations (1, 2, 4, 5, 6, 7, 8, and 16 mg/mL) of ethanolic extracts of propolis for 72 h. After treatment, MTT solution (20 µl) was added to each well and incubated for 4 h and then 100 mL of DMSO was added to each well. After 15 min, the color intensity of the wells was measured at 570 nm using a Biotek microplate reader. The results were expressed as percentage inhibition relative to control cells (considered as 100%). Logarithmic regressions were performed to define 50% inhibitory concentrations (IC50) (concentrations that inhibit the response by 50%). The experiment was repeated three times using different dilutions of the extracts.

Analysis of phenolic compounds by RP-HPLC

Fourteen standards of phenolic compounds were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC, (Elite LaChrom Hitachi, Japan) to determine propolis' phenolic profile. The sample was inserted into the HPLC system and installed with a reversed-phase C18 column (150 mm x4.6 mm, 5µm; Fortis). Water, acetonitrile, and acetic acid were applied for the mobile phase by using a programmed gradient. The mobile phase was formed of (A) 2% acetic acid in water and (B) acetonitrile in water (70:30). The injection volume of the samples was 20 µL, the column temperature was 30°C, and the flow rate was 0.75 mL/min. The programmed solvent used was maintained with a linear gradient held at 95% A for three minutes, decreasing to 80% A at 10 min, 60% A at 20 min, 20% A at 30 min, and finally started with a linear gradient decreasing to 95% A at 50 minutes.

Statistical analysis

A one-way test of variance (ANOVA) with the SPSS software package for Windows was applied to complete all statistical analogies. Mean ± standard error (SE) was used for all results. MTT data were analyzed using one-way ANOVA followed by the Student t-test. Differences between $p < 0.05$ were found to be statistically significant.

RESULTS

Amoebicidal activity

The trophozoite growth stopped in Turkish propolis ethanolic extracts with IC_{50}/48h at 5 mg/mL. Propolis showed stronger inhibitory effects at the concentrations of 7, 6, 5, 4, and 3 mg/mL with 72 h against *Acanthamoeba* trophozoites. The ethanol extracts of propolis reduced cell viability by approximately 2, 2.67, 22.67, 33.33, and 58.33% at concentrations 7, 6, 5, 4, and 3 mg/mL within 72 h, respectively. Among the different concentrations of propolis ethanolic extracts used in this study, 7 mg/mL propolis extract solution represented the strongest amoebicidal activity on trophozoites with IC50/72 h. High activity at low concentrations (3-7 mg/mL) of propolis extract can kill and can be an effective treatment for *Acanthamoeba* spp., after 72h treatment.

The effect of the propolis ethanolic extract on *A. castellanii* trophozoites is presented in Table 1, Table 2, and Fig. 1

The tests with propolis ethanolic extracts were done two times in triplicate. Results are presented as mean ± mean standard error (SE) in Table 1.

Table 1. Mean ± SE values for the percentages of cell viability of *Acanthamoeba* trophozoites when exposed to different concentrations of propolis extract for varied hours

Dose	*A. castellani* forms	Treatment periods (hours)						
		1 h	3 h	6 h	8 h	24 h	48 h	72 h
		Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE
7 mg/ml	Trophozoites	94.33±1.67	90.33±1.67	82.00±1.00*	65.00±1.00*	31.00±3.21*	3.33±2.33*	2.00±1.00*
6 mg/ml	Trophozoites	94.00±1.53	92.67±1.20	88.00±1.00*	75.00±0.58*	44.67±0.88*	32.50±3.50*	2.67±0.67*
5 mg/ml	Trophozoites	100.00±0.00	94.33±1.67	93.00±1.53	84.67±1.20*	66.67±2.40*	50.33±0.33*	22.67±1.20*
4 mg/ml	Trophozoites	100.00±0.00	100.00±0.00	94.33±1.67	89.00±1.00*	72.00±2.08*	56.00±2.08*	33.33±2.28*
3 mg/ml	Trophozoites	100.00±0.00	100.00±0.00	94.67±1.33	91.67±0.33	81.67±1.86*	69.00±2.00*	58.33±1.45*
1 mg/ml	Trophozoites	100.00±0.00	100.00±0.00	100.00±0.00	94.33±1.67	91.33±0.33	86.00±1.00*	75.33±2.91*

*= Statistic different from control group $p < 0.05$

Table 2. Percentages of cell viability in the *Acanthamoeba* trophozoites when exposed to 7, 6, 5, 4 mg/mL concentrations of propolis extract for 72 h.

The stage of *A. castellanii*	**The concentrations of propolis extract**	**Percentage of cell viability**
Trophozoites	7 mg/mL	2.00±1.00
	6 mg/mL	2.67±0.67
	5 mg/mL	22.67±1.20
	4 mg/mL	33.33±2.28

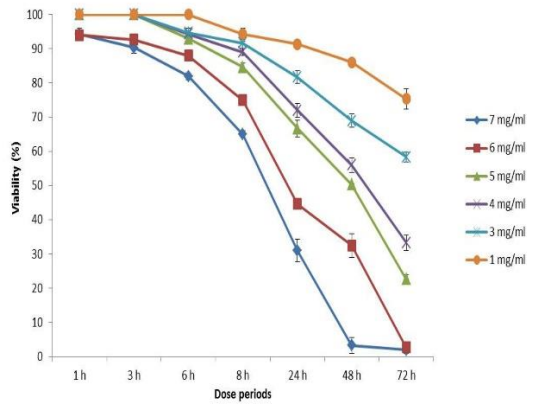

Figure 1. The effect of different concentrations of propolis extract on the cell viability of *A. castellanii* trophozoites at varying times (hours)

Propolis extract cytotoxicity

The effects of propolis extract on cell viability in BEAS-2B cells are shown in Figure 2.

The results of the MTT assay showed that propolis extract decreased cell viability in a concentration-dependent manner. These decreases in cell viability were significant at 4 mg/mL and above concentrations when compared to the control ($P<0.05$). The propolis's 50 % inhibitory concentrations

(IC50) were found as 6.60 mg/mL. Propolis showed stronger inhibitory effects at the higher concentrations tested (7, 8, and 16 mg/mL). The cell viability values of BEAS-2B cells were 43.28, 23.90, and 13.01 % at 7, 8, and 16 mg/mL concentrations, respectively. Based on our results, it can be stated that higher concentrations of propolis extract can induce cytotoxicity in BEAS-2B cells.

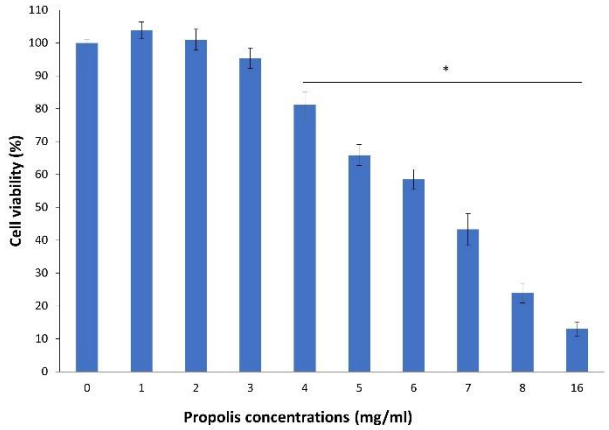

Figure 2. Effects of propolis on cell viability of BEAS-2B cells after 72 h treatment. *significant compared to control ($p < 0.05$).

Phenolic profiles

Fourteen standards of phenolic compounds were analyzed qualitatively and quantitatively using RP-HPLC-UV (Table 3). Protocatechuic acid, p-OH benzoic, Vanillic acid, Caffeic acid, Epicatechin, p-coumaric acid, Ferulic acid, Daidzein, t-cinnamic acid were present in differing amounts in samples investigated, while Caffeic acid, Catechin, Gallic acid, Syringic acid, Rutin, Luteolin were not detected in propolis. The major phenolic compound in propolis was Ferulic acid, and lower levels of Protocatechuic acid, Caffeic acid, and p-OH benzoic acid were also detected.

Table 3. Phenolic profiles of propolis extract collected from different regions in Turkey

Standard	***Results lg phenolic compound /g sample***
Gallic acid	n.d.
Protocatequic acid	0.024
p-OH benzoic acid	0.087
Catechin	n.d.
Vanillic acid	5.575
Caffeic acid	0,025
Syringic acid	n.d.
Epicatechin	2.220
p-coumaric acid	0.569
Ferulic acid	13.215
Rutin	n.d.
Daidzein	2.142
t-cinnamic acid	3.505
Luteolin	n.d.

**n.d: not detected.*

DISCUSSION

Propolis is a resinous hive product collected by honeybees from various plant sources. Propolis has an extensive variety of biological activities because of its flavonoids and caffeic acid phenethyl ester (16). It has recently been given as a dietary supplement for the therapy of various diseases (17).

Many studies have suggested that the therapeutic properties of propolis the anti-bacterial (4), anti-inflammatory (18), anti-viral (19), anti-tumoral (5), anti-fungal (20), anti-oxidant (21), anti-protozoal (22), activities.

These activities of propolis samples correlate with the total phenolic contents. The caffeic acid derivatives and flavonoids of propolis have strong antimicrobial activity. Thus, there is increasing interest in it as the origin of new therapies (23). However, we know that limited studies in Turkey have been reported on propolis's anti-amoebic and cytotoxic properties so far.

Several studies on the amoebicidal activity of propolis against *Acanthamoeba* species originated in Turkey. Topalkara et al. (7) first reported the propolis effect on *Acanthamoeba* trophozoites and cysts. In their study, there was a decrease in the number of viable trophozoites of *A. castellanii* based on a dose-dependent application of propolis. Propolis (higher than 8 mg/mL) had a lethal efficacy on *Acanthamoeba* trophozoites, while no lethal concentration of propolis for *Acanthamoeba* cysts after 1 h incubation. A second study was reported by Vural et al. (24) on the propolis effect in a rat model of *Acanthamoeba* keratitis and assigned it is in vitro cytotoxic activity in cultured corneal epithelial cells. This study showed the propolis extract had an anti-amoebic effect in this rat model of *Acanthamoeba* keratitis. Following these studies, we aimed to show propolis's cytotoxic and amoebicidal activities collected from different regions in Turkey.

For this reason, propolis extracts with different concentrations were analyzed to indicate their efficiency against the trophozoites of *A. castellanii*. The trophozoites were inhibited with 7mg/mL of propolis extract with a 2.00±1.00 percentage of cell viability at 72 h. Propolis extract (7 mg/mL) showed stronger amoebicidal activity on trophozoites since there were fewer viable trophozoites in this mixture at 72 h.

Previous studies by Duran et al. (25) indicated that the ethanolic extract of propolis samples in Adana from Turkey showed a remarkable antileishmanial effect on *Leishmania tropica*. In this study, the maximum reduction in the proliferation of *L. tropica* parasites was detected in cultures exposed to 250, 500, and 750 µg/mL of propolis. In addition, antileishmanial activities of Bursa and Hatay propolis samples against *Leishmania infantum* and *Leishmania tropica* strains were detected with the gas chromatography-mass spectrometry technique

by Duran et al. (26). The results showed that these propolis samples reduced remarkably the multiplication of *L. infantum* and *L. tropica* parasites. The chemical compounds of propolis samples were examined by high-resolution GC-MS. They reported that these propolis samples contain a high concentration of compounds such as aromatic acids, aromatic acid esters, flavanols, and cinnamic acid esters. They found some types of propolis had more antileishmanial effects than others against *Leishmania* species because of the various geographical sites, regional flora, and plant variety.

Similarly, many studies were reported on antibacterial (27) antiviral (23,28) antifungal (29) and, in antiparasitic (30) properties of propolis related to the presence of flavonoids and phenolic compounds and chemical composition in the propolis.

The result of the analysis of active components in propolis in different studies is quite different since the composition and contents of these active components depend on different factors including season and vegetation of the field, propolis collection techniques, and geographical origin (31)

Many studies have been reported to analyze the phenolic compounds of propolis from different regions in Turkey (31,32,33). The present study is similar to those of our previous studies in showing the phenolic compounds of propolis collected from different regions in Turkey. However, according to the analysis of the phenolic compounds in the present study, the propolis samples found high concentrations of Ferulic acid and Vanillic acid.

Ferulic acid is completely spread in plant cell walls and has anti-inflammation, anti-diabetic, anti-oxidation, and antiviral effects (28). In addition, Celińska-Janowicz et al. (34) reported the polyphenols of propolis such as ferulic acid, chrysin, p-coumaric acid, and caffeic acid induce anti-proliferative activity by increasing the role of proline metabolism and proline dehydrogenase/proline oxidase, active caspases-3, and -9 expressions, P53, and decrease prolidase activity, proline concentration and, collagen biosynthesis in CAL-27 cells.

Vanillic acid is one of the main pharmacologically active molecules in propolis due to its ability to have anti-inflammatory, anti-metastatic, antidiabetic, antioxidant, cardioprotective, and anti-apoptotic effects (35). Kılıç Altun and Aydemir (31) reported that the amount of Vanillic acid among all phenolic ingredients of propolis was the highest level after Hydroxycinnamic acid, Kaempferol, and Quercetin. The amount of vanillic acid level in propolis in our study is similar to those of the other previous studies. There were high concentrations of Ferulic acid.

Cytotoxic effects of some propolis samples from Turkey on different human cells have been reported in previous studies.

Anticarcinogenic effects of propolis have been shown in human lymphocytes culture (36), and peripheral blood lymphocytes (37). Ethanolic extract of Turkish propolis showed proapoptotic activity and antiproliferative effect on the human lung cancer (A549) cell line with 31.7 µg/mL IC50 value (38). The anticarcinogenic effects of the propolis samples were reported on aggressive cell lines SK-BR-3, MDA-MB-231, and nonaggressive breast cancer cell line (BCCL) MCF-7 (39). Some ethanolic extract of Turkish propolis displayed a higher cytotoxic activity against A549 lung cancer cells (IC50=25.44 µg mL^{-1}) when compared to BEAS-2B healthy lung epithelial cell cultures (IC50=55.68 µg mL^{-1}) (40). The differences in IC50 values in the studies may be due to the differences in the regions where the samples were collected, the extraction methods used, the solvents, the management of the cell cultures, the stages of the experiments, and the treatment times used. We wanted to see the response of high concentrations of propolis samples in healthy cells in the present study. We found that the extract of propolis did not show significant cytotoxic effects up to a concentration of 3 mg/mL. Based on the results of previous studies, we can say that its cytotoxicity is probably higher in lung cancer cells. It is very important to protect the healthy cells because of higher cytotoxicity in cancer cells and lower toxicity in healthy cells.

CONCLUSION

Acanthamoeba infection is still a problematic parasitic disease that is not fully treated with drugs. In this study, we found that propolis extract collected from different regions in Turkey has major amoebicidal and cytotoxic activities. These activities could widely be interesting in pharmaceutical areas. Propolis extract can be thought of as a natural product that can be used against *Acanthamoeba* trophozoites. Even though further in vivo studies are needed to show the real effect of propolis extracts on *Acanthamoeba* infections, these results may be alternative therapeutic drug sources for the treatment of acanthamoebiasis alongside existing drugs.

Ethics Committee Approval: Ethical approval is not required for this study

Author Contributions: Concept: Z.K, Ö.E, Z.K, Ü.K, Design: Z.K, Ö.E, Z.K, Ü.K, Literature search Z.K, Ö.E, Z.K, Ü.K, Data collection and Processing: Z.K, Ö.E, Z.K, Ü.K, Analyses and Interpretation: Z.K, Ö.E, Z.K, Ü.K, Writing: Z.K, Ö.E, Z.K, Ü.K,

Conflict of Interest: There are no conflicts of interests related to this study.

Financial Disclosure: There are no external funding sources for this study.

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