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RESEARCH ARTICLE

Evaluation of Anthelmintic Effects of Essential Oil of Star Anise Against *Ascaridia Galli* of Poultry

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ABSTRACT

Ascaridia galli is among the most reported nematode helminths leading to severe losses in terms of reduced productive and reproductive performance of poultry. Essential oils have been reported to control helminths effectively because of their unique phytochemicals. Thus, star anise essential oil was evaluated for its *in vitro* and *in vivo* anthelmintic activity. Phytochemical analysis was performed using gas chromatography flame ionization detection assay. For *in vitro* efficacy study, egg hatch assay, larval motility, and worm motility assays were performed. Fecal egg count reduction test was performed to evaluate efficacy of selected essential oil *in vivo*. During the *in vivo* experiment, blood parameters (RBC, PCV, Serum protein) were also assessed. The phytochemical analysis revealed that the star anise essential oil has Anetholes (Cisa and anethole) as major phytochemical compound. The *in vivo* efficacy was determined using three different concentrations of star anise essential oil (10, 20 and 30mL/kg in the feed). The essential oil was effective in a dose-dependent manner, with the maximum concentration showing a significant difference ($P<0.05$) compared to the negative controls. Egg hatchability, worm and larval motility were significantly ($P<0.05$) reduced *in vitro*. A significant reduction ($P<0.05$) was observed in fecal egg counts, along with improvements in RBC, PCV and serum protein levels. These results demonstrate that star anise essential oil can be effectively used for the control of *A. galli* in poultry.

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INTRODUCTION

Poultry is the major sector that plays an important role in global food security and the economy. It is facing several issues including infectious agents, which are putting constraints on the growth of this sector (Bachaya *et al.*, 2015; Saeed *et al.*, 2023). Gastrointestinal (GI) helminths are among the most prominent pathogens of livestock and poultry (Saeed and Alsayeqh, 2023). *Ascaridia (A.) galli is* a nematode parasite found in the intestine of the broiler and layer birds (Feyera *et al.*, 2022b). It is a commonly transmitted nematode which can be acquired through direct ingestion or by transport hosts like earthworms (Ritu *et al.*, 2024). The larvae of *A. galli* are aggressive blood feeders and may lead to severe signs such as decreased weight gain, enteritis, blood, sugar loss and increased nitrogenous contents (Shohana *et al.*, 2023). The eggs of *A. galli* can also be present in the eggs of the chickens. Because of these issues, the control of *A. galli* is among the issues in the poultry sector (Feyera *et al.*, 2022a).

Chemically synthesized anthelmintics are commonly used for the control of *A. galli* in commercial poultry flocks (Jilo *et al.*, 2022). Currently, resistance is being reported against routinely used anthelmintics in parasites including *A. galli* (Saeed and Alkheraije, 2023). In this scenario, alternative control strategies are urgently needed to treat developing infections (Abbas *et al.*, 2023). Additionally, the concerns have been raised regarding the potential public health impacts of anthelmintic drugs. The increasing demand for organic poultry solely relies on natural control measures. The chemical-free production of poultry necessitates the development of natural alternatives for the control of *A. galli* (Shohana *et al.*, 2023).

Herbal remedies are promising ways to control parasites due to their perceived safety, efficacy, and potential for sustainable and accurate application (Ur-Rehman *et al.*, 2023). Various herbal formulations are being investigated as potential treatments for nematodes including *A. galli*. Essential oils are volatile compounds comprised of lipophilic components with low molecular weights and a volatile nature (Al-Hoshani *et al.*, 2023).

They are among the most bioactive constituents of plants and have demonstrated anthelmintic, antiprotozoal, immunomodulatory, and anti-inflammatory properties in numerous studies.

Star anise is a plant native to various parts of the world, including the subcontinent. It is an important culinary ingredient (Al-Hoshani *et al.,* 2023). It is also widely used for its aromatic and medicinal properties. Numerous studies have reported the antiparasitic, anthelmintic, immunomodulatory, and anti-inflammatory potential of star anise, including its essential oil (Patra *et al.,* 2020). Based on these observations, this study aims to determine the phytochemical composition of star anise essential oil and evaluate its *in vitro* and *in vivo* anthelmintic activity against *A. galli* in chickens.

MATERIALS AND METHODS

Plant material and essential oil extraction: Star anise seeds were obtained, air-dried, ground into a fine powder and soaked in water. Subsequently, steam distillation was performed in distillation flasks for 6 hours. The essential oils were separated using a volatile solvent and anhydrous sodium sulfate was used to remove impurities. The solvent was then removed using rotary evaporation at 60°C following the methods of Saeed *et al*. (2023).

Phytochemical assay: The essential oil extracted was then analyzed using Gas Chromatography-Flame Ionization Detection (GC-FID) on a SHIMADZU® apparatus equipped with a DB-WEX 30mx0.25mm column. Nitrogen was used as the carrier gas at a flow rate of 20ml per minute. All procedures were performed according to the methods described by Ali *et al*. (2019).

Parasite collection: Adult motile worms were collected from layer, breeder, nondescript and organic poultry farms within the locality. Briefly, suspected infected birds were slaughtered and their GI tracts were examined for the presence of *A. galli*. Intestines containing adult worms were collected and transported to the laboratory under sterile conditions. The worms were identified microscopically and placed in sterile pathogen-free containers. Female worms were separated from males to facilitate egg collection. All procedures were conducted according to standard protocols of Katakam *et al*. (2010).

Egg embryonation assay: Eggs of *A. galli* were collected from adult female worms isolated from the intestines of chickens obtained from local farms in South Punjab, specifically district Rahim Yar Khan. Six groups of eggs were established, each consisting of three replicates, with 100 eggs per replicate. These eggs were isolated in suspension and incubated in four different concentrations of essential oil as 1.25, 2.5, 5 and 10mg/mL, respectively. A standard medicated control group containing 10mg/mL of albendazole was included and a negative control group containing phosphate-buffered saline was also established. All eggs were incubated at a temperature of 30±2°C for 12 days. All procedures were performed according to standard protocols as described by Coles *et al*. (1992).

Larvicidal activity: *In ovo* larvicidal efficacy of star anise essential oil was evaluated against unshelled second-stage larvae (L2) of *A. galli*. The group formation, dosage of star anise essential oil and controls were same as described for egg embryonation assay. 50 larvae per group were exposed to the treatments. After 12 hours, the number of non-motile (no movement observed within 10 seconds) and motile larvae were counted. Percent motility was calculated according to standard methods of Coles *et al*. (1992).

Anthelmintic activity against adults: Freshly collected adult *A. galli* worms were maintained in Ringer-Locke solution. The group formation, dosage of star anise essential oil and controls were same as described for egg embryonation assay. Each group consisted of three replicates, with 10 worms per replicate. The worms were exposed to treatments for 24 hours at a temperature of 30±3°C and observed at 6-hour intervals. All experimental procedures were conducted according to standard protocols form Sen *et al*. (2020).

***In vivo* experiment:** A total of 150 one-day-old COBB-500® chicks were purchased from a commercial hatchery and housed on the farm. The vaccine schedule, lighting, and housing conditions were maintained according to the COBB broiler production guide (Tavernari *et al*., 2013). A standard commercial broiler diet was provided, as outlined in Table 1. On the 15th day of age, the birds were randomly divided into five groups, each consisting of three replicates with 10 chicks per replicate. Groups 1 to 3 received 10, 20 and 30mL/kg (1, 2 and 3 v/w), respectively, of star anise essential oil in their feed. Group 4 received albendazole at a dose of 10mg/kg in their feed as a positive control, while Group 5 served as an infected unmedicated control. Each group was experimentally infected with 500 *A. galli* eggs per bird. Fecal samples were collected daily from each group and examined for the presence of *A. galli* eggs. The day on which *A. galli* eggs were first detected in the feces of any group was designated as Day 0 for egg count analysis. Adult birds exhibiting egg counts of 800±50 eggs per gram of feces (EPG) were selected for further experimental infections.

Table 1: Composition of the feed used in the current experiment.

Ingredients	Quantity (%)
Corn	44
Rice (grain)	12
Rice (polishing)	4
Canola meal	15
Soybean meal	11
Molasses	3
Corn gluten meal (60%)	4
Fish meal	4
Di-calcium Phosphate	2
Vito mineral mixture	0.65
DL-methionine	0.10
L–lysine	0.25

Fecal egg count reduction test: Fecal egg counts were performed weekly for two weeks and the EPG of feces was determined for each sample following the methods of Vadlejch *et al*. (2011). Percent fecal egg count reduction was estimated by the methods described by Saeed and Alsayeqh (2023).

Fecal egg count reduction (%) = (Initial worm counts-final worm counts/Initial worm counts) *100

Hematology and serum protein estimation: Serum protein, PCV and RBC levels were determined at the end of the experiment according to the method described by Ahmad *et al*. (2022). RBC counts were determined using the method of Natt and Herrick (1952). Serum protein levels were quantified using spectrophotometric methods (Saeed *et al*., 2023).

Statistical analysis: Statistical analysis was performed using Minitab 26.0 statistical software. General linear model was applied for analysis of variance and Tukey's post hoc test was used for comparison of means. Statistical significance was determined at the 5% confidence level (P<0.05).

RESULTS

Phytochemical assay: GC-FID analysis of star anise essential oil revealed the presence of more than 10 compounds (Fig. 1). The top 10 compounds are listed in Table 2. Trans-anethole and cis-anethole were the major components, comprising 19.6% and 17.8% of the oil, respectively. Multiple other terpene derivatives were also identified (Fig. 1; Table 1).

Egg embryonation assay: The unembryonated eggs were kept for 12 days in various treatments (1.25, 2.5, 5, and 10% v/v) of essential oil and the results were checked based on percent embryonation. Only 10% concentration of the star anise essential oil and albendazole (10 mg/kg) had a 100% stop to embryonation and were significantly (P<0.05) different from all other groups (Table 3).

***In vitro* larvicidal activity:** The unshelled L2 were kept for 24 hours in various treatments (1.25, 2.5, 5, and 10% v/v) of essential oil and the results were checked based on percent embryonation. Only albendazole (10mg/kg) had 100% inhibition of larval mortality if *A. galli* 10% and concentration of the star anise essential oil were statistically comparable (P>0.05) to it. Both medicated control and the 10% essential oil group were significantly different (P<0.05) from all other groups (Table 3).

***In vitro* vermicidal activity:** The adult worms of *A. galli* were kept for 24 hours in various treatments (1.25, 2.5, 5 and 10% v/v) of star anise essential oil and the results were checked based on percent motility. There was no embryonation at 5 and 10% concentrations of the star anise essential oil and in albendazole treated group. Results were significantly (P<0.05) different from all other groups (Fig. 2).

Fecal Egg count reduction: The EPG of feces were performed on days 0, 7, and 15 of post-fecal excretion of Ascaridia sp. eggs. The data were analyzed for a weekly and biweekly reduction in the egg counts. Maximum reduction was observed in standard medicated control having a comparable relation to the 3% concentration of star anise essential oil (Table 5).

Hematology and serum protein: Effects on RBC, PCV and serum proteins were evaluated. The results confirmed that the star anise essential oil significantly increased the RBC, PCV and serum protein levels, which were comparable to infected medicated control and significantly (P<0.05) different from the other groups (Table 5).

DISCUSSION

Resistance to commonly used therapeutic antibiotics is among the most serious challenges facing researchers in this decade (Church and McKillip, 2021). Infectious agents, including helminths, are rapidly developing resistance, leading to the failure of synthetic drugs within a short timeframe. Researchers have reported the urgent need for alternative therapeutic strategies and have proposed phytochemicals as ideal substitutes due to their multidimensional modes of action (Ashraf *et al.*, 2023).

Table 2: Phytochemical composition of star anise essential oil evaluated through Gas Chromatography Flame Ionization Detection Technique

Sr. No	Name of Component	Retention Time (Minute)	Concentration(%)
1.	Trans -anethole	47.9	19.6
2.	Cis-anethole	41.0	17.8
3.	Gamma-terpineol	23.3	10.1
4.	Linalool	37.6	9.6
5.	Alpha-Phellandrene	19.4	8.1
6.	P-Cymene	5.12	7.5
7.	alpha-Phellandrene	32.8	6.7
8.	Beta-Pinene	16.72	5.3
9.	Beta myrcene	2.3	5.0
10.	Limonene	30.84	3.7

Table 3: Egg embryonation and larval motility of *A. galli* worm treated with various treatments of star anise essential oil compared to controls

Concentration of essential oil (mg/mL)	The egg embryonated (%)	Larval motility (%)
3.125	64.33 ±3.93^{bc}	67.37±8.96^{b}
6.25	42.73±5.6^{d}	53.1±2.72^{c}
12.5	30.87±5.05^{e}	36.91±3.22^{d}
25	0.00±0.0^{f}	2.33±1.33^{ef}
Positive control	0.00±0.0^{f}	0.00±0.0^{f}
Negative control	97.37±0.59^{a}	95.91±0.59^{a}

Values with the same superscripts have statistically comparable results (P>0.05)

Table 4: Eggs per gram and percent fecal egg count reduction (%FECR) of *A. galli* on a weekly interval in the chicken

Groups	Eggs Per Gram (% FECR)		
	Day 0 (% FECR Week 1)	Day 7 (% FECR Week 2)	Day 15 (% FECR Total Period)
A	833.33±104.08^{a} (21.68±4.05)	650±50^{a} (46.33±3.57)	350±50^{b} (58.04±1.87)
B	800±132.28^{a} (29.25±18.7)	550±50^{a} (38.53±17.19)	333.33±76.37^{bc} (58.63±2.99)
C	866.67±104.08^{a} (61.42±1.6)	333.33±28.86^{b} (75.39±6.87)	83.33±28.86^{c} (90.56±2.41)
D	816.66±104.08^{a} (59.02±1.99)	333.33±28.86^{b} (60.31±5.49)	133.33±28.86^{c} (83.8±1.72)
E	-	-	-

Values with the same superscripts have statistically comparable results (P>0.05).

Table 5: Percent packed cell volume (PCV), red blood cells (RBC) and total proteins in the chicken infected with *A. galli*

Treatment	PCV (%)	RBC (x10^{6}/uL)	Total proteins (g/dL)
A	31.49±0.86^{a}	3.16±0.21^{a}	3.05±0.16^{b}
B	33.3±1.91^{a}	3.32±0.13^{ab}	3.1±0.13^{b}
C	33.48±0.88^{a}	3.45±0.09^{b}	3.5±0.28^{a}
D	24.67±0.88^{b}	3.43±0.08^{b}	3.43±0.1^{a}
E	34.26±0.89^{a}	2.98±0.12^{a}	3.0±0.23^{b}

Values with the same superscripts have statistically comparable results (P>0.05)

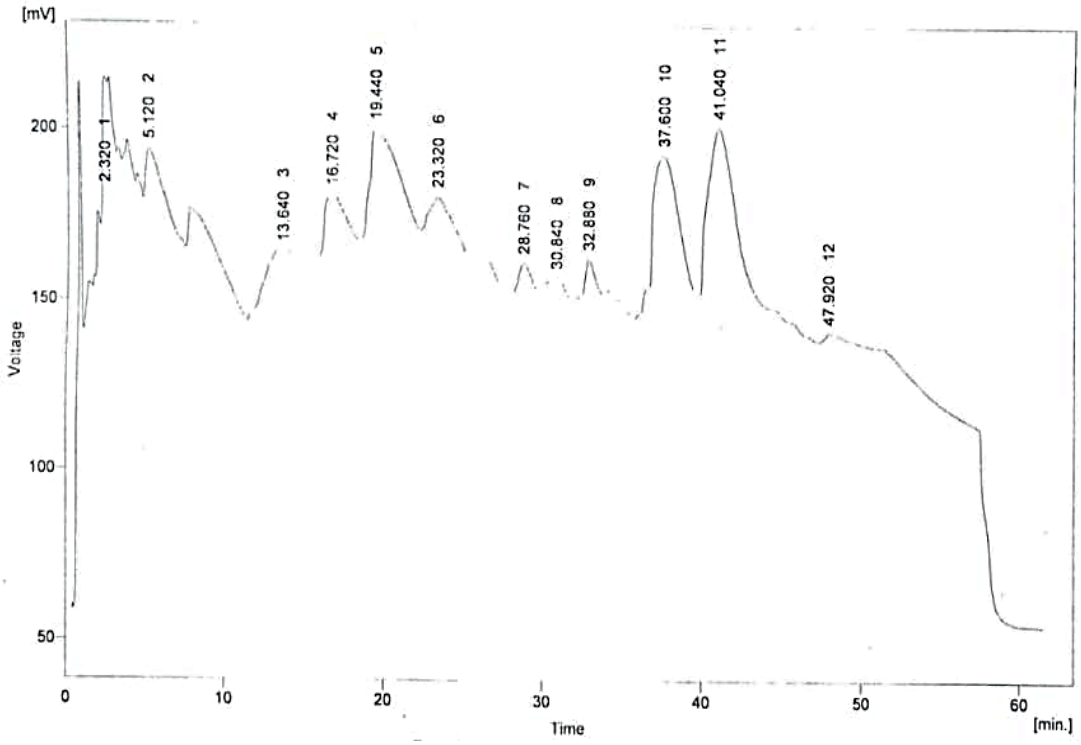

Fig.1: Chart of Phytochemical composition of star anise essential oil evaluated through Gas Chromatography Flame Ionization Detection Technique.

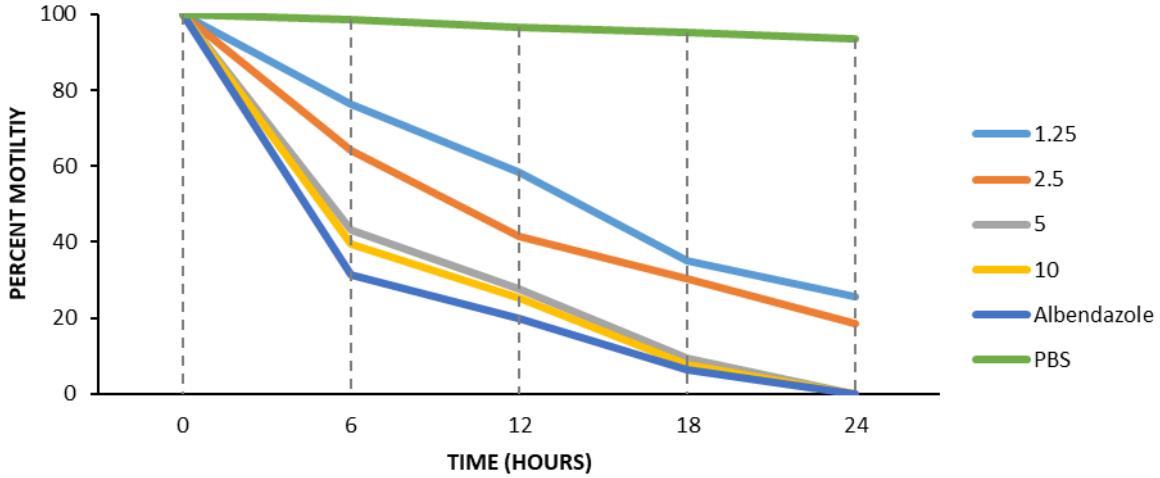

Fig. 2: Percent motility of adult *A. galli* exposed to various concentrations of essential oil of star anise (mg/mL) compared to albendazole and phosphate buffer saline (PBS) in *in vitro* experiment.

Plants contain a diverse array of chemical compounds, among which terpenes and their derivatives hold significant importance (Li *et al.*, 2023). These compounds are lipophilic and are commonly extracted as fatty fractions, such as essential oils (Dajic Stevanovic *et al.*, 2020; Zielińska-Błajet and Feder-Kubis, 2020).

In a current study, GC-FID analysis of the star anise essential oil revealed that anetholes were the major constituents. Other terpenes, terpenoids, and terpinols were also frequently detected in the oil (Table 1). Previous studies have also reported a similar composition for star anise essential oil (Shahrajabian *et al.*, 2021; Sharafan *et al.*, 2022; Soonwera *et al.*, 2024). Minor variations in the concentration of these compounds can occur due to factors such as geographic origin, cultivar, extraction method, and analytical techniques used for phytochemical analysis (Alirezalu *et al.*, 2020; Boukhatem and Setzer, 2020). The presence of these compounds supports the observed anthelmintic activity of anise essential oil against *A. galli* in our study.

Terpenes and their derivatives, including cis-anethole, trans-anethole, terpineol and other derivatives, are well-known for their anthelmintic properties in both *in vitro* and *in vivo* studies (Silva *et al.*, 2021; Torres-Fajardo and Higuera-Piedrahita, 2021; Panda *et al.*, 2022). These compounds show their anthelmintic effects through various direct and indirect mechanisms. Studies have shown that terpenes can disrupt the integrity of the helminth eggshell, leading to reduced embryonation (Serra *et al.*, 2022; Espino Ureña *et al.*, 2023). Furthermore, research indicates that compounds present in essential oils can inhibit egg embryonation by interfering

with *in-ovo* metabolic activities. This can involve disrupting the nutrition of developing embryos and acting as antagonists to essential enzymes such as proteases and chitinases, which are crucial for the bioavailability of the components within the eggshell (Sousa *et al.,* 2021; Mohamed *et al.,* 2024). These mechanisms likely contribute to the reduced egg hatchability observed in our study (Table 3, Fig. 2). Similar results have been reported in other studies evaluating the anthelmintic effects of essential oils on egg embryonation and larval viability (Štrbac *et al.,* 2022; Štrbac *et al.,* 2023). Minor variations in efficacy among different essential oils can be attributed to variations in their terpene composition.

Essential oils have larvicidal and vermicidal activities through direct activities because of several mechanisms. The active components of essential oils can kill the larvae and adults of the helminths by the same mechanisms of action because of similar morphologies despite differences in nutritive efficiencies (Helal *et al.,* 2020; Matté *et al.,* 2023). The control of larvae of *A. galli* is more important than the adults because the larvae are the most aggressive feeders and are responsible for blood and protein loss (Feyera *et al.,* 2022a). Moreover, the first two stages, L_1 and L_2 develop inside the eggs and the third instar is hatched inside the body. The essential oils can kill the larval stages by disturbing the metabolism, nutritional intake blockage, cuticle disruption, and many other functions (de Carvalho Augusto *et al.,* 2020; Matté *et al.,* 2023). Because of these activities, essential oils have been reported to control nematode larvae, including *A. galli.* This study's results align with previous experiments (Helal *et al.,* 2020; Wang *et al.,* 2020). Because of similar modes of action, vermicidal activities can be justified by with same rationale, and similar results have been reported in our study and previous research. Essential oils are volatile fractions and evaporate easily in *in-vitro* conditions (Houdkova and Kokoska, 2020), so there are less than expected larvicidal activities *in vivo* experiments, however, statistically comparable ($P>0.05$) results pertaining to standard control were reported. This activity finds its justification because of direct exposure and increased concentration of the oils.

The terpenes and terpenoids of essential oils control the helminth egg count by a similar mechanism except that they also reduce the reproductive performance of adults (Ramdani *et al.*, 2023; Băieș *et al.,* 2024) and cause a reduction in egg production and fertilization through several known and unknown mechanisms. Moreover, the terpenes and terpenoids help control the infection because of their indirect immunomodulatory and anti-inflammatory mechanisms (Saeed and Alkheraije, 2023). Although several other factors, including intestinal environment, microbial load metabolism, etc. can affect the potency of essential oils in *in-vivo* environments (Ramdani *et al.*, 2023; Băieș *et al.,* 2024) similar effects are reported which are reported in *in vitro* studies. Therefore, a similar trend of percent fecal count was observed and the essential oil of star anise had comparable effects at maximum concentration to standard medicated control while significantly different results from the non-medicated control.

The RBC, PCV and serum proteins were greatly improved and had significantly different results from infected non-medicated control. The RBC and protein values were improved because larvae feed on blood and disturb the blood cells and protein levels (Helal *et al.,* 2020). So, the larval and adult count was reduced, and these parameters were improved. All the other parameters remained normal, which indicates the essential oil had no toxic effects on the hepatorenal functions.

Conclusions: This research concludes that the star anise essential oil can be used for the control of *A. galli* in poultry and can reduce the larva and worm motility in the chicken. The effective concentration of the star anise was 30mg/mL which shows promising results. Although no resistance was observed in the research against standard medicine, the star anise can be a suitable alternative if resistance is reported.

Authors contribution: MR and ASMA were actively involved in research designing, execution, data analysis, data interpretation and write up of the manuscript. Both authors approved the final version for publication.

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