Significant effects of two pesticides on the bacteriostatic activity and antioxidant ability of green tea polyphenols

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Title page

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Abstract

Background: Green tea polyphenols (GTPs) have good bacteriostatic activity and antioxidant capacity, yet pesticide pollutants in tea may affect their functionality. This study aims to explore the effects of pesticide pollutants on the bacteriostasis and antioxidant ability of GTPs.

Results: The bacteriostatic activity of GTPs and two pesticides (acetamiprid (ACE), diquat dibromide (DIQ)) shows some certain time characteristics. Two pesticides can affect the bacteriostatic activity of GTPs. The bacteriostatic activity of GTPs is enhanced or weakened by the two pesticides with time lengthening, i.e. time-dependent synergism or antagonism. The bacteriostatic mechanisms of the three substances and their mixtures is produced by affecting cell morphology or destroying cell structure, and the long-term antagonism of the three substances is may due to the competition of action site. In addition, the two pesticides can greatly reduce the antioxidant capacity of GTPs. ACE reduces the free radical scavenging ability of GTPs by 14%~24% and DIQ reduces the free radical scavenging ability of GTPs by 39%~63% at the experimental concentration ratios.

Conclusions: Two pesticides has significant effects on the bacteriostatic activity and antioxidant ability of GTPs.

Key words: Green tea polyphenols; Pesticides; Bacteriostatic activity; Mechanism; Antioxidant capacity

Background

Tea has become a necessity for many people. As we all known, green tea polyphenols (GTPs) accounting for 15%~30% of the dry weight of tea are the general name of polyphenols with catechin as the main component in tea, and have good bacteriostatic activity [1,2]. Ben Lagha et al. [3] pointed out that GTPs can produce bacteriostatic toxicity to *Fusobacterium nucleatum* by damaging cell membrane. Cho et al [4] found that GTPs can affect the cell morphology and structure of some oral bacteria.

In addition, GTPs have strong antioxidant capacity apart from bacteriostatic activity [5-7]. Therefore, a proper amount of GTPs can not only delay the aging of the human body, but also can protect the genetic material DNA of the human body from being damaged by free radicals in the process of copying [8]. This means that the loss of antioxidant capacity of GTPs will lead to the loss of its health efficacy.

However, many pesticides are used in tea to promote production. Thus, pesticides have become common residual pollutants in tea. Yi et al. [9] tested 390 tea samples on the market, and found that 52% of the samples had pesticide residue problems. Kobayash et al. [10] investigated the pesticide residues of 116 imported tea samples in Tokyo market, and found that the detection rate of pesticides in

unfermented tea was 90%. Therefore, Pesticides probably coexist with tea functional components, GTPs. Then, whether the residual pesticide pollutants in tea will affect GTPs' bacteriostasis activity and antioxidant capacity is a matter of concern.

Therefore, this study aims to explore the effects of pesticide pollutants on the bacteriostasis and antioxidant ability of GTPs. To do so, two commonly used pesticides (acetamiprid (ACE), diquat dibromide (DIQ)) in tea were selected as research objects. A freshwater luminous bacteria *Vibrio qinghaiensis sp.*-Q67 (Q67) which is very sensitive to toxic substances was selected as test organisms. The bacteriostatic ability data of GTPs, two pesticides and their binary mixtures to Q67 were determined by using time-dependent microplate toxicity analysis method (t-MTA) [11-14]. The binary mixture system of GTPs and two pesticides was constructed by using direct equipartition ray design method (EquRay) [15]. And the concentration addition model (CA) with 95% observation-based confidence interval (OCI) was used to evaluate the interaction action between pesticides and GTPs [16-20]. The bacteriostatic mechanism of the three substances and their mixtures to Q67 was preliminarily determined by observing the cellular morphology [4,21]. Besides, free radical scavenging ability of binary mixture rays was determined by the method of salicylic acid [22]. The results would provide a data reference for healthy drinking of tea.

Materials and methods

Chemicals

GTPs (Green tea polyphenols) were purchased from Shanghai Yuanye

Biotechnology Co., Ltd (Shanghai, China). Two pesticides, acetamiprid (ACE) and diquat dibromide (DIQ), were purchased from the national pesticide quality supervision and testing center of Shenyang chemical research institute (Shenyang, China). All the reagents were of analytical grade and used as received without further purification. The physical and chemical properties of the three reagents, the concentration of the stock solution, and the dilution factor (*f*) are listed in Table 1. The storage solution was prepared with Milli-Q water and stored at 4 ℃.

(Table 1 around here)

Bacterial culture

The freeze-dried luminescent bacterium *Vibrio qinghaiensis sp.*-Q67 (Q67) was purchased from Beijing Hamamatsu Corp., Ltd. (Beijing, China). The preparation of the culture medium and culture process of Q67 are detailed in the literature [12].

Determination of bacteriostatic activity

The bacteriostatic data of GTPs, two pesticides and their binary mixtures to Q67 were obtained by t-MTA. Using 96 microporous plate as experimental carrier, the relative luminous unit (RLU) of each hole were measured at 2 h, 4 h, 8 h and 12 h, respectively, and the luminous inhibition toxicity of single components and its mixtures to Q67 were calculated by Eqn(1) to reflect the bacteriostatic activity of the pollutants to Q67 at each time point. The design of the microplate is detailed in the literature [11,12].

$$x\% = \frac{I_0 - I}{I_0} \times 100\% \quad (1)$$

where I_0 is the average RLU of blank control group, I is the average RLU at each

concentration gradient.

Experimental design of mixtures

Binary mixture system of GTPs and two pesticides was constructed by using direct equipartition ray design method (EquRay) [15]. Each mixture system contains five rays with different concentration ratios (p_is), and each ray was diluted to 12 fixed specific concentration points according to the dilution factor obtained by the pre-experiment [12]. The p_is of each component of binary mixtures are shown in Table 2.

(Table 2 around here)

Concentration-effect curve fitting

The bacteriostatic data obtained by t-MTA were fitted by Logit (Eqn(2)) or Weibull (Eqn(3)) functions. The fitting and the calculation of 95% observation-based confidence interval (OCI) were completed by APTox software [23]. The two function formulas are as follows:

$$E = 1/(1+\exp(-\alpha-\beta*\log_{10}(c))) \quad (2)$$

$$E = 1-\exp(-\exp(\alpha+\beta*\log_{10}(c))) \quad (3)$$

where E represents the effect ($0 \le E \leqslant 1$), c represents the concentration of a single compound or mixture, α and β represent model parameters.

Effects of two pesticides on bacteriostatic activity of GTPs

The interaction between GTPs and two pesticides was evaluated by using the relatively conservative CA model [16-20,24]. The calculation was completed by APTox software [23]. The function is as follows:

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x,i}} = 1 \quad (4)$$

where n is the number of mixture components, $EC_{x,i}$ the concentration of the ith component that provokes $x\%$ effect when applied individually, and c_i the concentration of the ith component in the mixture.

Bacteriostatic mechanism to Q67

In order to investigate the bacteriostatic mechanism of GTPs, two pesticides and their binary mixtures on Q67, the cell morphology of Q67 exposed to GTPs, two pesticides and their binary mixtures at the concentration of EC_{80} for 12 h was measured. Firstly, Q67 bacterial suspension of logarithmic growth period was placed in conical flask. Then, GTPs, two pesticides and their binary mixtures were added to the conical flask, respectively, so that the concentration of drugs in the suspension was equal to the EC_{80} of GTPs, two pesticides and their binary mixtures at 12 h. Lastly, the conical flask was cultured in a constant temperature incubator at 22 ±1 ℃ for 12 h. After 12 h, the cell morphology of Q67 was observed by scanning electron microscope. The specific steps for the preparation of electron microscope samples refer to the relevant literature [4,21].

The effects of two pesticides on the antioxidative ability of GTPs

The free radical scavenging ability of GTPs and two pesticide binary mixtures under different p_is (experimental group) were determined by the method of salicylic acid [22]. Then, replace pesticides in binary mixtures with equal volume Milli-Q water as control (blank group) to determine the free radical scavenging ability of GTPs. The greater the difference between the free radical scavenging ability of the

experimental group and the control group is, the greater the effects of pesticides on the antioxidant capacity of GTPs.

Results and discussion

Bacteriostatic activity of individual components to Q67

The nonlinear least square method was used to fit the concentration - response data of GTPs and two pesticides to Q67 at 0.25 h, 2 h, 4 h, 8 h and 12 h. The results show that the Weibull function can effectively characterize the concentration-response relationship of GTPs, ACE and DIQ ($R>0.9$, $RMSE<0.1$). The specific fitting results and relevant statistic parameters are shown in Table 1. The time-concentration-response curves (t-CRCs) of single components are shown in Fig.1.

From Fig.1, the time characteristics of the bacteriostatic activity of GTPs and two pesticides to Q67 are different. The bacteriostatic activity of GTPs increases with the prolongation of exposure time within 0.25 ~ 12 h, while the bacteriostatic activity of DIQ decreases within 0.25 ~ 2 h, but increases within 2 ~ 12 h. The bacteriostatic activity of ACE to Q67 is not affected by exposure time, and its t-CRCs almost coincide at five exposure time points. Combined with Table 1, taking pEC_{50} ($-lgEC_{50}$) as the bacteriostatic activity index, the bacteriostatic activity order is as follows at 0.25 h and 2 h: ACE (pEC_{50}=3.44 ~ 3.47) > GTPs (pEC_{50}=3.17 ~ 3.26) > DIQ (pEC_{50}=3.81 ~ 3.85). The bacteriostatic activity order is as follows at 4 h,8 h and 12 h:DIQ (pEC_{50}=3.81 ~ 6.79) > GTPs (pEC_{50}=3.61 ~ 4.14) > ACE (pEC_{50}=3.55 ~ 3.60).

In conclusion, the antibacterial effect of GTPs and two pesticides on Q67 varies

with the exposure time [25]. And it is noted that in addition to the good bacteriostatic activity of GTPs, two pesticides, as toxic substances, also have inhibitory toxicity to bacteria due they are pesticides with high toxicity to nontarget organisms [26,27].

(Fig.1 around here)

Effects of two pesticides on bacteriostatic activity of GTPs

Weibull function can also effectively characterize the concentration-response relationship of binary mixture rays of GTPs and two pesticides($R>0.9$，$RMSE<0.1$). The fitting results and relevant statistic parameters are shown in Table S1. The t-CRCs of binary mixture rays are shown in Figure S1. The results of the CA prediction are not always in agreement with the experimental results, and when the CA prediction curve is higher or lower than the OCI, there is an interaction between the components of the mixture [16-20,28].The experimental observations of each mixture ray with obvious synergism at different time points and its 95% OCI, fitting curves and CA prediction results are shown in Fig. 2 and Fig. 3. (Other results are drawn in Figure S2).

(Fig.2 around here)

(Fig.3 around here)

It can be seen from Fig. 2 and Fig. 3 that the five rays of the GTPs-ACE binary mixture system show synergism and additive action in exposure times of 0.25 h and 2 h, and the CA prediction line is located below or between the OCI. However, with the prolongation of exposure time, five mixture rays all show antagonism, and the CA prediction line is located above the OCIs. The five mixture rays of GTPs-DIQ binary

mixture system show synergism when the exposure time is 0.25 h, but with the prolongation of exposure time, the toxicological interaction changes from synergism to antagonism. Therefore, the toxicological interaction of the two groups of binary mixtures tends to antagonism with the prolongation of exposure time.

So, from the results of toxicity interaction analysis, it can be seen that ACE and DIQ can affect the bacteriostatic activity of GTPs, and the influence mode differs in different exposure time. The short-term synergism of the mixture system will strengthen the bacteriostatic ability of GTPs, while the long-term antagonism of the mixture system will lead to the decrease of bacteriostatic ability of GTPs. Therefore, pesticide pollutants will affect the functionality of GTPs from the point of view of bacteriostatic activity.

The bacteriostatic mechanism to Q67

The cell morphology of Q67 exposed to the EC_{80} of GTPs, two pesticides and their representative rays for 12h is shown in Fig. 4. From Fig. 4, the morphology of Q67 cells is changed compared with the control group. GTPs and DIQ make Q67 cells bond with each other and destroyed the membrane structure of bacteria, which make the contents of Q67 flowed out. While ACE prolonged Q67 cells and ruptured Q67 cells. The rays with the greatest long-term bacteriostatic activity in two binary mixture systems were selected to observe their effects on morphology of Q67 cells. It is found that mixtures also damaged the morphology of Q67 cells. Besides, it is found that the amount of bacteria in the experimental group is lower than that in the control group after centrifugation. This suggests that GTPs, two pesticides and their mixtures

194 can inhibit the reproduction of bacteria. Cho et al [4] also found that tea polyphenols
195 could destroy the cell structure of the bacteria. Therefore, the three substances may
196 have similar antibacterial mechanisms, and the long-term antagonism of the three
197 substances is due to competition of action site [29].
198 (Fig.4 around here)

199 **Effects of two pesticides on the antioxidative ability of GTPs**

200 In order to characterize the effects of two pesticides on the antioxidative ability
201 of GTPs, the free radical scavenging ability of each binary mixture ray was
202 determined by salicylic acid method. The results are shown in Fig. 5. As can be seen
203 from Fig. 5, the free radical scavenging ability of each binary mixture ray is lower
204 than that of the control group. ACE reduces the free radical scavenging ability of
205 GTPs by 14%~24% and DIQ reduces the free radical scavenging ability of GTPs by
206 39%~63% at the experimental concentration ratios. This indicates that pesticide
207 pollutants can damage the antioxidant capacity of GTPs, and the extent of damage is
208 DIQ > ACE. Therefore, antioxidant capacity of GTPs will be greatly affected by
209 residual pesticides, which will affect the quality of the tea by weakening GTPs'
210 functionality.
211 (Fig.5 around here)

212 ## Conclusion

213 GTPs and two pesticides all have antibacterial activity, and its bacteriostatic
214 activity shows some certain time characteristics. Both ACE and DIQ can affect the
215 bacteriostatic activity of GTPs. The bacteriostatic activity of GTPs will be enhanced

when the two pesticides and GTPs coexist for a short time. However, the bacteriostatic activity of GTPs will be weakened when the two pesticides and GTPs coexist for a longer time. That is to say, mixtures of the two pesticides and GTPs exhibit time-dependent synergism or antagonism. The bacteriostatic mechanism of GTPs, two pesticides and their mixtures on Q67 is produced by affecting cell morphology or destroying cell structure. Therefore, GTPs and two pesticides may have similar antibacterial mechanisms, and the long-term antagonism of the three substances is due to the competition of action site. In addition, the two pesticides can greatly reduce the antioxidant capacity of GTPs, and the extent of damage is DIQ > ACE.

Abbreviations

GTPs: Green tea polyphenols; ACE: Acetamiprid; DIQ:Diquat dibromide; Q67: *Vibrio qinghaiensis sp.*-Q67; t-MTA: Time-dependent microplate toxicity analysis method; EquRay: Direct equipartition ray design method; CA: Concentration addition model; OCI: 95% observation-based confidence interval; RLU: Relative luminous unit; t-CRCs: Time-concentration-response curves.

Declarations

Acknowledgments

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Authors' contributions

The corresponding author JZ is responsible for ensuring that the descriptions are accurate and agreed by all authors. The author MT is responsible for investigation and writing -original draft, and ZH is responsible for formal analysis. GH is responsible for resources, and SZ is responsible for supervision.

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Availability of data and materials

All data supporting the conclusions of this article are included within the article and one additional file.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

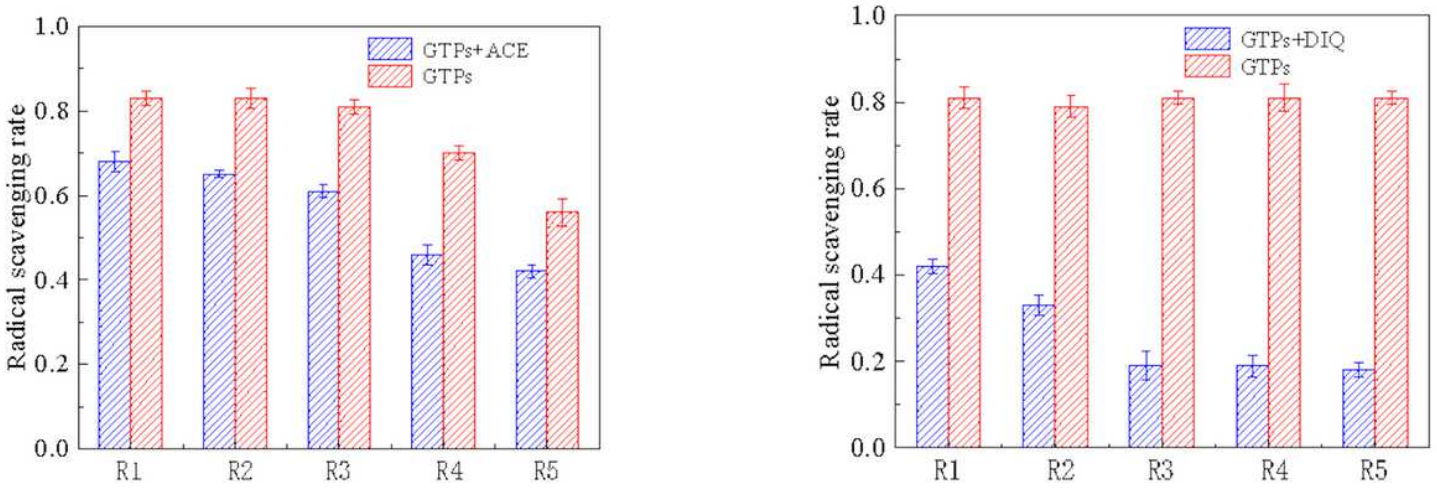

Figure 1

The t-CRCs of GTPs and two pesticides.

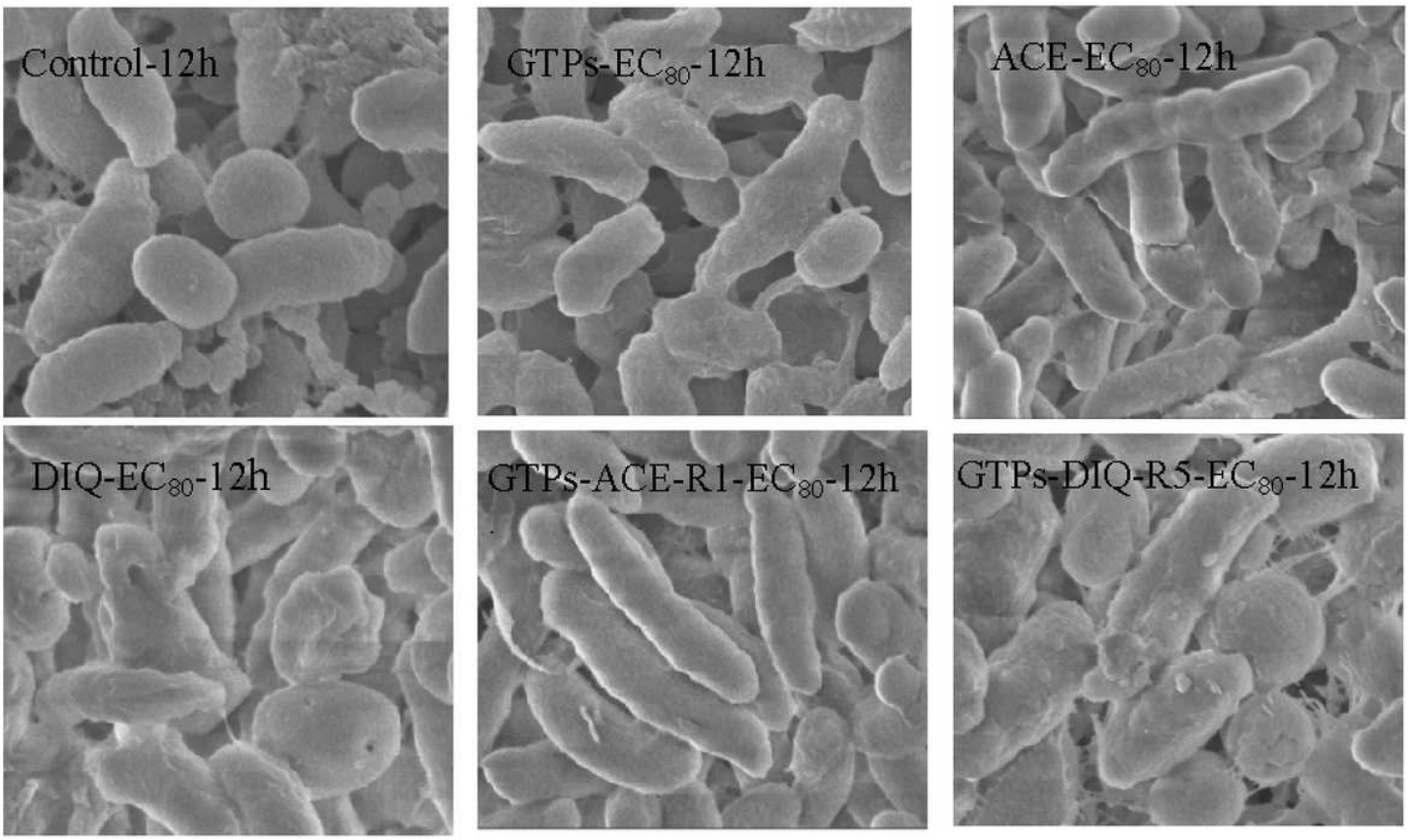

Figure 2

The observed concentration-effect data with 95% OCIs, fitted CRCs and predicted curves by CA of representative rays with synergism or antagonism for GTPs-ACE mixture systems (□: observed data; —:

fitted curve; —: predicted curve by CA; -▯-: 95% OCI).

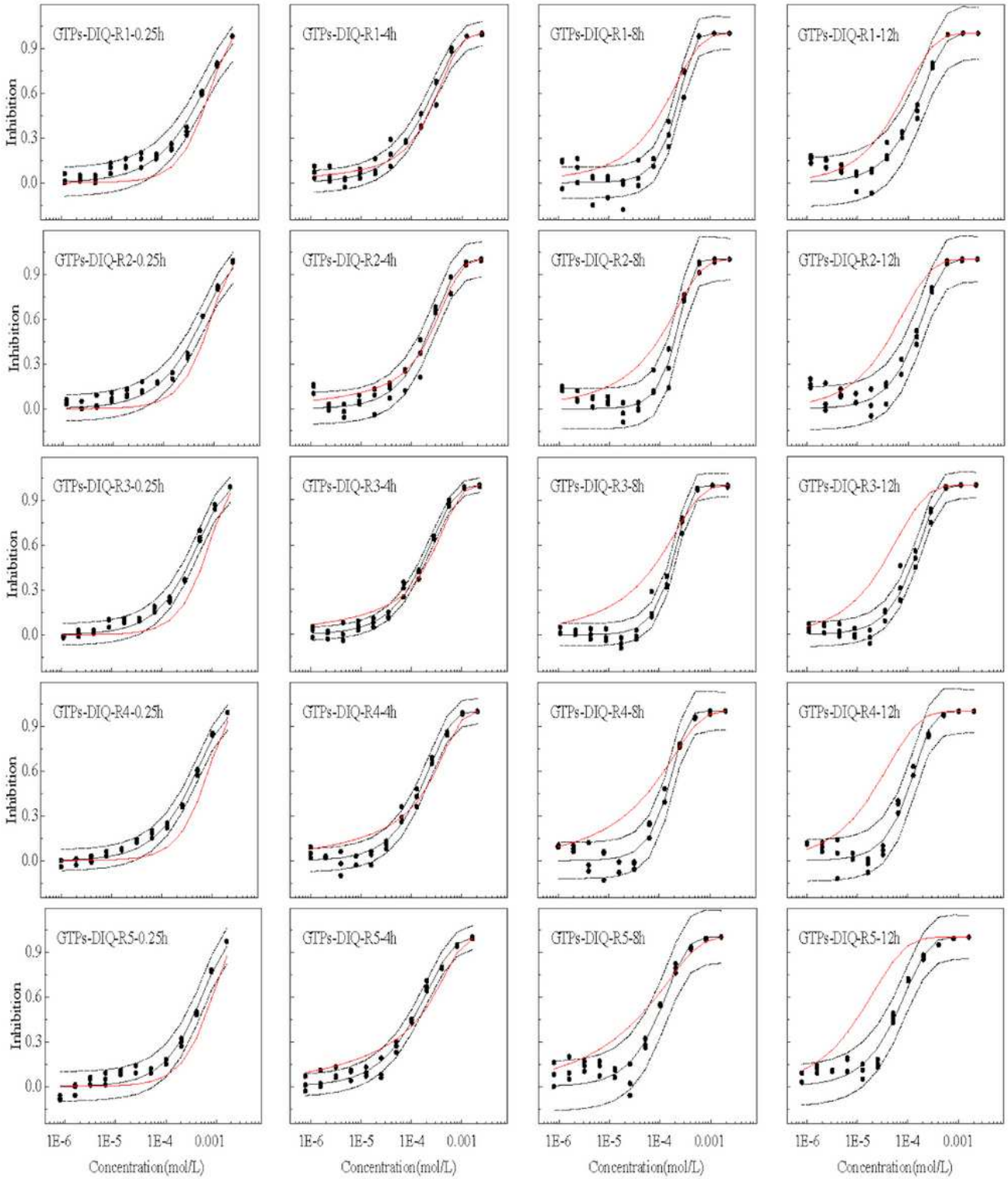

Figure 3

The observed concentration-effect data with 95% OCIs, fitted CRCs and predicted curves by CA of representative rays with synergism or antagonism for GTPs-DIQ mixture systems (▯: observed data; —: fitted curve; —: predicted curve by CA; -▯-: 95% OCI).

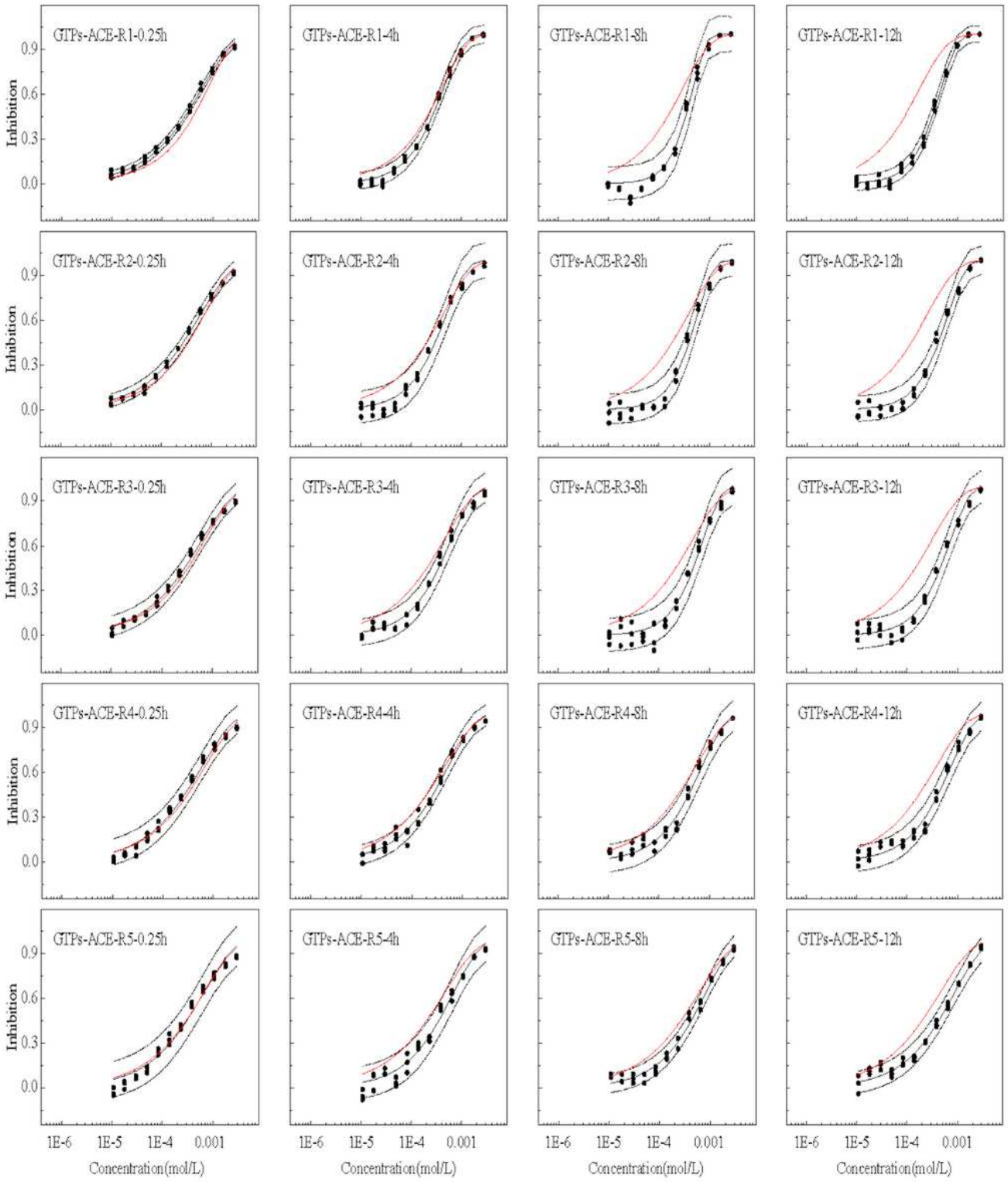

Figure 4

The cell morphology of Q67 exposed to the EC80 of GTPs, two pesticides and their representative rays for 12 h.

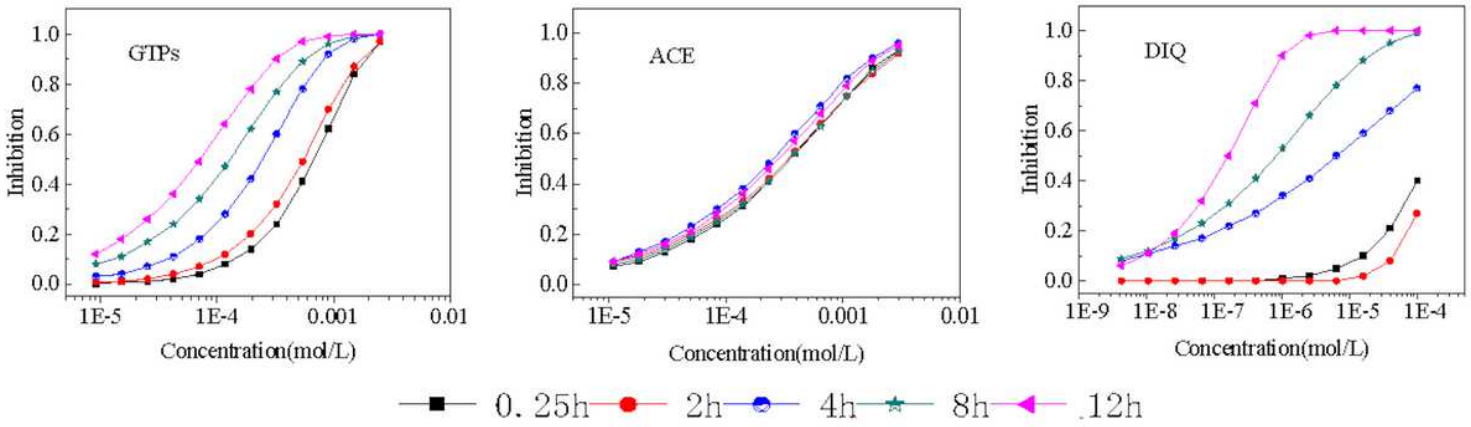

Figure 5

Effects of ACE and DIQ on GTPs radical scavenging ability under different concentration ratios.

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