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In silico toxicity of the natural pesticides clitoriacetal and 6-deoxyclitoriacetal: an ecotoxicological strategy

Toxicidade in silico dos pesticidas naturais clitoriacetal e 6-desoxiclitoriacetal: uma estratégia ecotoxicológica

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ABSTRACT

Brazil has a position of visibility in the popularization of pesticides which has caused public and environmental health problems. Thus, the search for new natural insecticides that meet appropriate guidelines is found in plants, as is the case of the compounds clitoriacetal and 6-deoxyclitoriacetal isolated from plants of the genus *Clitoria* constituted of rotenoids with insecticidal activity. In this context, this study aims to investigate the *in silico* toxicity of the pesticides clitoriacetal and 6-deoxychlitoriacetal in A. melifera bees, enabling the biosafety of these organisms. Based on the *in silico* models: BeetoxAI© (acute oral toxicity) and molecular docking a criteria for biochemical evaluation. In this way, it was established that the derivatives clitoriacetal and 6-deoxycyclitoriacetal have oral toxicological potential (acute) due to the various hydrogen acceptor sites and low quantitative exposure value. And from the molecular docking, it is pointed out that the complexes formed between the ligands and protein of *A. melifera* present similar three-dimensional positions but do not express any interaction with amino acids of the binding site of *A. melifera* conferring them low toxicity. The study was developed in an initial character, that is, it will still be necessary to improve and deepen the techniques applied and expand new *in silico* and *in vivo* assays.

KEYWORDS: *In silico;* ecotoxicology; *Apis melifera*; natural pesticides.

RESUMO

O Brasil possui uma posição de visibilidade na popularização de pesticidas no qual tem ocasionado problemas de saúde pública e ambiental. Assim, a busca por novos inseticidas naturais que atendam diretrizes adequadas são encontrados nas plantas, como é o caso dos compostos clitoriacetal e 6-desoxiclitoriacetal isolados de plantas do gênero *Clitoria* constituídos de rotenóides com atividade inseticida. Nesse contexto, o objetivo deste estudo consiste na investigação de toxicidade *in silico* em abelhas *A. melífera* dos pesticidas clitoriacetal e 6-desoxiclitoriacetal viabilizando a biossegurança destes organismos. Baseando-se nos modelos in silico: BeetoxAI© (toxicidade aguda oral) e docking molecular como critério de avaliação bioquímica. Dessa forma, foi estabelecido que os derivados clitoriacetal e 6-desoxiclitoriacetal apresentam potencial toxicológico por via oral (agudo) devido aos diversos sítios de aceitadores de hidrogênio e baixo valor quantitativo de exposição. E a partir do docking molecular pontua-se que os complexos formados entre os ligantes e proteína da *A. melífera* apresentam posições tridimensionais similares, mas não exprime nenhuma interação com aminoácidos do sítio de ligação da *A. melífera* conferindo-os baixa toxicidade. O estudo foi desenvolvido em caráter inicial, ou seja, ainda serão necessário melhoria e aprofundamentos das técnicas aplicadas e ampliações de novos ensaios *in silico* e *in vivo*.

PALAVRAS-CHAVE: *In silico;* ecotoxicologia; apis melífera; pesticida naturais.

INTRODUCTION

From agricultural modernization began a process of rapid, reckless, and early industrialization of pesticides in agropastoral areas. This was based on high dependence on chemical fertilizers, mechanization of crops, selection of transgenics with high production potential, wide dissemination of irrigation and drainage systems, as well as dependence on economic and fiscal incentive policies for the promotion and development of contemporary agriculture (COSTA & PIRES 2016, RAMOS et al. 2018).

This way, Brazil has stood out in the world import/export of cereals, fruits, and other products of vegetable origin, as a result of this increase it was indispensable to insert insecticides, organic or inorganic, to control, kill and repel pests in crops (AGUIAR et al. 2019). However, the continuous and incorrect use of pesticides can offer risks to terrestrial and aquatic organisms, as well as occupational poisoning caused in surrounding areas by the use of these agents through consumption and ingestion of water or via contaminated food (ARAÚJO & OLIVEIRA 2016).

In this context, the search for new insecticides that meet the requirements of efficacy, biosafety, and selectivity is found in studies of plant defense mechanisms. Natural products are sources of several commercialized pesticides and can be applied in the form of extracts or pure compounds extracted from microorganisms, plants, and animals, many active structures can be used as prototypes for the synthesis of new pesticides (HALFELD-VIEIRA et al. 2016).

Some plant extracts have been used by man until the present day, but few of these, including those containing pyrethrins, terpenes, and alkaloids have been used as sources of insecticides (FERREIRA et al. 2022, VIEGAS-JÚNIOR 2003). Thus, rotenoids and rotenones are also a class of natural products biosynthesized from seeds and roots of plants of the genus *Leguminosa* found in various tropical regions of the world (DE MOURA & SCHLICHTING 2007, SANTOS et al. 2016).

In their chemical composition, rotenoids consist of a four-fused tetrahydrochromeno[3,4b] chromene ring core skeleton, which is a modified isoflavone with an extra carbon atom in the heterocyclic system (INÁCIO 2007) (Figure 1), where each member of the ring core can be specifically substituted for several groups, enabling the variability of biological activities such as anti-inflammatory, antifungal, antioxidant, insecticide, and others (PITAKPAWASUTTHI et al. 2021, PK et al. 2021).

Nevertheless, the rotenoids clitoriacetal (Figure 1A) and 6-deoxyclitoriacetal (Figure 1B) can be extracted from the seeds and roots of *Clitoria* plant using the methodology of DE PASSOS et al. (2019) and BERTONCELI et al. (2022) through drying and grinding, then the extracts were subjected to retro-evaporation and condensation with MeOH:H2O solvents (1:3 *v*/*v*) and partitioned with CH_2Cl_2, EtOAc, and n-butanol, where its easy extraction enables its dissemination in the chemical-biological industry.

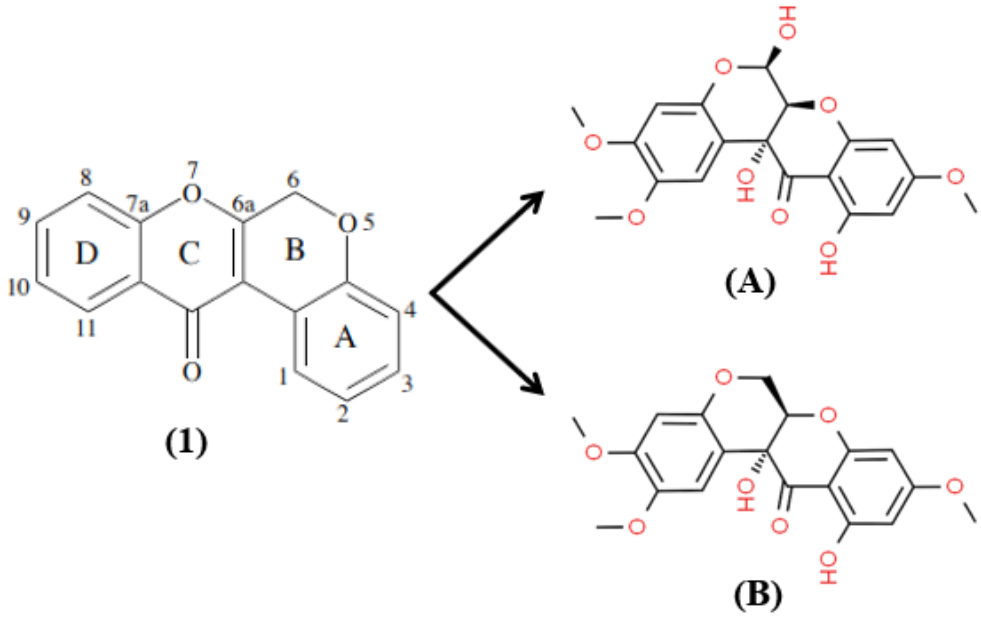

Figure 1. Basic structure of rotenoids. **(A)** Molecular structure of clitoriacetal e **(B)** 6-deoxyclitoriacetal.

Thus, rotenoids are presented as promising prototypes for extraction, synthesis, and commercialization, given their high insecticidal potential, and low persistence in the environment, due to their physicochemical properties (INÁCIO 2007). In the ecotoxicological context, rotenoids show low toxicity in mammals, but have lethargic or toxic activity for fish, and some classes of insects (DE MOURA &

SCHLICHTING 2007).

Due to the absence of studies on toxicity in insects of the genus *Apis* that are pollinating agents of various wild plants and crops. Chemically induced toxicity can cause the extinction of these bees resulting in several threats to food security and ecosystem services. Thus, the registration and re-evaluation systems of regulatory agencies have required specific acute toxicity tests on bees (NOCELLI et al. 2012).

Thus, *in silico* predictive studies can assist researchers in studies and evaluation of the effects of natural or synthetic substances on living organisms in their ecosystems, including the forms of transport of these substances and their interactions with the environments. Since these tests require low costs and high accuracy potential due to their mathematical and statistical models propose predictive strategies to the *in vivo* test (CARVALHO & PIVOTO 2011).

Thus, the objective of the present work was to investigate the toxicological profile in the acute and biochemical state of the natural pesticides clitoriacetal and 6-deoxyclitoriacetal using as bioindicator *Apis mellifera* correlating their physicochemical properties, aiming at the biosafety of the organism due to its sensitivity to chemicals, given from an *in silico* approach aiming to reduce future impacts on human and environmental health.

MATERIAL AND METHODS

BeetoxAI©

BeeToxAI is an artificial intelligence QSAR (Quantitative Structure-Activity Relationship) tool developed to assess the acute toxicity of chemicals in honey bees. The models showed high predictive power achieving specific accuracy, sensitivity, and variance. The BeeToxAI web server has an intuitive interface that allows the user to perform predictions quickly and efficiently (ALVES et al. 2018, ZHENG et al. 2021).

Still in their studies ALVES et al. (2018) describe that the contribution maps correspond to the prediction of atoms and fragments that collaborate for acute contact and oral toxicities. This method provides a simple interaction of the predicted results, helping researchers to propose structural modifications aimed at reducing the acute toxicity potential of chemicals (MOREIRA-FILHO et al. 2021).

Docking Molecular

Initially, natural pesticides are inserted in geometric and structural optimization which consists of a molecular modeling technique, which aims to work the three-dimensional structure of the ligand to find specific coordinates where its lowest energy state is determined, and thus, consequently working with a more stable compound, due to the minimization of potential energy (PAULING & WILSON 2012).

The structural optimization of the ligand adopted the parameters established with the classical force field MMFF94 (Merck Molecular Force Field 94) (HALGREN 1996). To perform the calculations, the Avogadro® software was used, where it had its configuration was established following the parameters for the use of MMFF94, after the conclusion of the simulation, the structure of the clitoriacetal (CTA) and 6-deoxyclitoriacetal (6-DCTA) ligands were obtained in three-dimensional dimension with its energy potential properly minimized and consequently more stable (HANWELL et al. 2012).

The dimeric crystallized structure of a pheromone-binding protein (PBD) from *A. melifera* in complex with 9-keto-2(*E*)-decanoic acid (9-ODA) at pH 7.0 was also obtained through the virtual platform Protein Data Bank - PDB identified with code (PDB ID: 3CYZ), presenting a resolution equivalent to 1.80Å (PESENTI et al. 2009).

The 9-ODA protein structure consists of a set of enzymes that act on pheromone receptors in the sensory membranes of queen bees of the species *A. melifera* that provoke sexual and social responses, as well as the identity profile of bees (workers and drones) (PESENTI et al. 2009).

In addition, the choice of the protein under study was based on the experimental data snapshot (EDS) characteristics (method and resolution) and specific protein action profile in bees of the species *A. melifera*. However, the choice of 9-ODA is unprecedented, since there are no studies showing protein structures for predictive ecotoxicology studies for this organism.

In addition, the use of the gridbox implies and delimits the regions of action of the ligand, that is, it is essential they gridbox values that establish an adequate scope and must go around the entire protein region aiming at the coupling of the protein during the simulation. Thus, the gridbox values are center_x = 23.129, center_y = -9.103, center_z = -11.52, size_x = 98, size_y = 104, size_z = 126, spacing = 0.442 and exhaustiveness = 8, using the AutodockTools® software (SHITYAKOV & FÖRSTER 2014).

The molecular docking simulations were performed with the aid of the AutoDockVina® software (GAILLARD 2018), for a better obtaining of results we selected the results that obeyed the reference

parameters, within the literature, such values are free energy of binding (ΔG) equivalent to or less than -6.0 kcal/mol (YUSUF et al. 2008), and results of RMSD (Root Mean Square Deviation) equal to or less than 2.0 Å (SHITYAKOV & FÖRSTER 2014). Based on some studies and observations of various ligand-protein interactions, we can highlight that distances between 2.5 and 3.1 Å are considered strong, from 3.1 to 3.55 Å are medium interactions and >3.55 Å are weak (IMBERTY et al. 1991).

RESULTS AND DISCUSSION

BeetoxAI©

Regarding toxicity using Apis bees as a biomarker, it can be seen that Figure 2A (CTA) and 2B (6-DCTA) express with the *Random forest* algorithm the creation of a random decision tree consisting of the final choice of acute oral toxicity in bees.

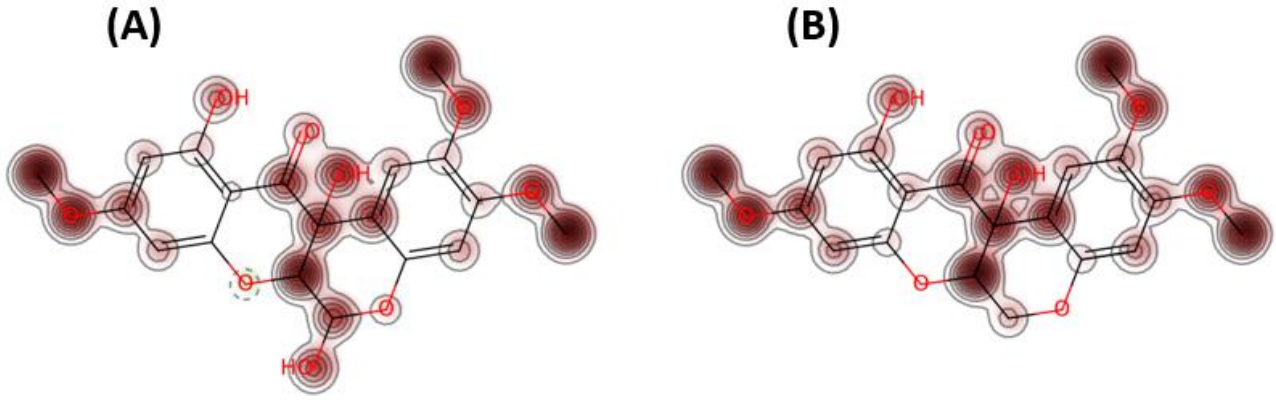

Figure 2. Evaluation of acute oral toxicity in *A. melifera* bee (A) CTA and (B) 6-DCTA.

Thus, the target compound (A) CTA establishes oral toxicological potential with quantitative value at $LD50 = 7.853\mu/bee$ ($pLD50 = 5.10 \pm 0.14$) with intrinsic probability at 63%, (B) 6-DCTA shows the same behavior scoring toxicological response value with intrinsic probability at $LD50 = 9.544\mu/bee$ $pLD50 = 5.02 \pm 0.14$ with an intrinsic probability of 61%, which may be related to values < 11.0µ/bee and the many hydrogen acceptor sites, i.e. bees will be affected in short-term exposures.

This is exemplified by REGES et al. (2019) and SANTIAGO DE OLIVEIRA et al. (2019), who demonstrated from semi-empirical calculations that clitoriacetal and its derivative 6-deoxyclitoriacetal present susceptible areas of nucleophilic connections that make up the entire oxygen area and variation of charges between atoms of the same species.

In a predictive way, one can initially correlate the toxic potential in oral (acute) mode as a result of the hypersensitivity of bees when exposed to any toxicants, where they can generally be associated with latency of movements, imbalance in their ecosystem functions, and interactions of these in the hives, but not necessarily death, as they are responses to short exposures.

Molecular Docking

Given the role of the 9-keto-2(*E*)-decanoic acid (9-ODA) receptor in caste selectivity and bee organization, the possible inhibition of this protein may cause an imbalance in the communication and reproductive cycle of bees, so it was necessary to analyze the biochemical mechanisms between pesticides and selective proteins of *Apis*.

In this context, the computational simulation was performed where data were obtained regarding the complexes formed between the ligands clitoriacetal and 6-deoxyclitoriacetal with the dimeric crystallized structure of the pheromone of the bee *A. melfera* (PDB ID: 3CYZ), as well as the results of free energy of binding (ΔG), binding distance between ligands and amino acids, and their respective RMSD values. With the study of the protein in question, the amino acids of references were analyzed: VAL 13.A; VAL 13.B; ILE 119.A; ILE 119.B; LYS 17.A and LYS 17.B. (PESENTI et al. 2009).

Thus, the ligand clitoriacetal in complex with the characteristic protein of *A. melifera* presents values of the free energy of binding, which is equal to -6.8 kcal/mol and the value referring to the RMSD of 1.851Å, these values are within the established parameter, where the energy value must be less than or equal to -6.0 kcal/mol and the RMSD must be less than 2.0Å.

It is observed in Figure **3 (A)**, a three-dimensional structure, which represents the formation of the complex between the ligand clitoriacetal (CTA- Blue), in which all the possible existing interactions between

the ligand clitoriacetal, with the protein in question, are demonstrated, where it is clear the presence of the following amino acids, residues, and chain: GLY B:72 with two carbon and hydrogen type bonds; ASP B:68 with one hydrogen type interaction; SER A:57 with two hydrogen type interactions and ASP A:66 with one bond with carbon and hydrogen characteristics.

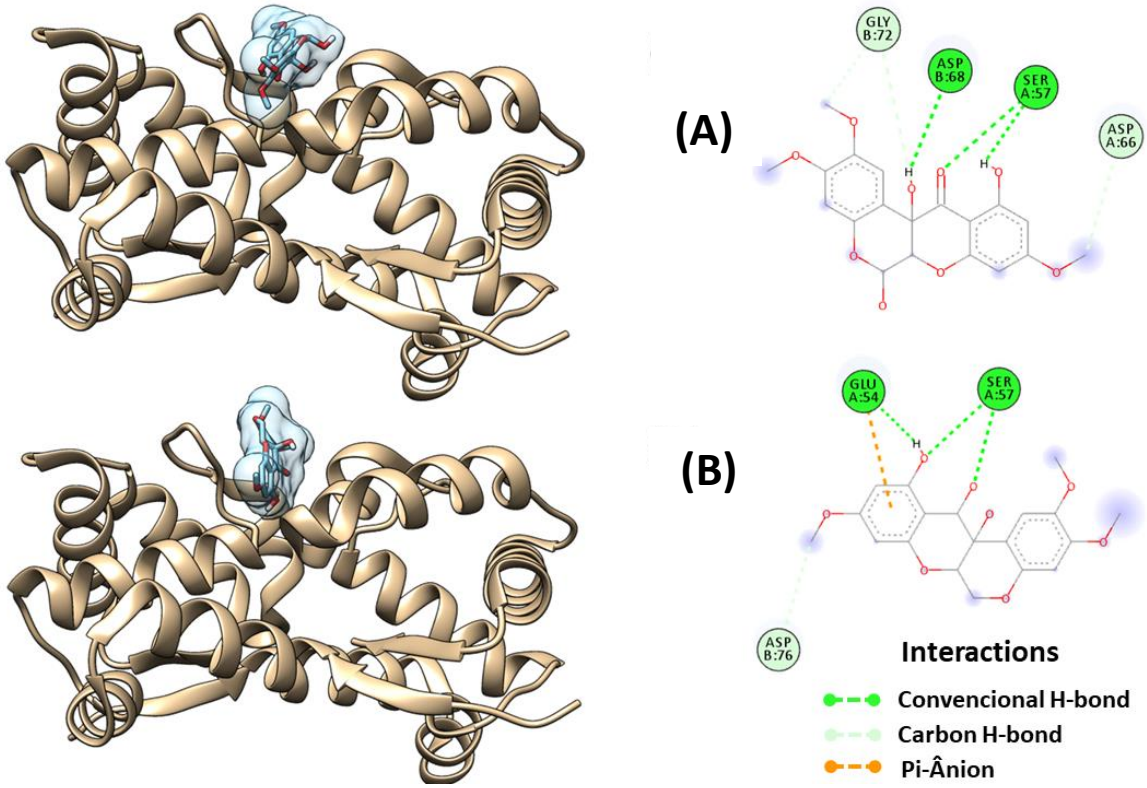

Figure 3. (A) complexed CTA compound and amino acid interactions with A. mellifera protein (B) complexed DCTA and ligand interactions.

Nevertheless, the complex originating between the ligand 6-deoxyclitoriacetal points out that the value of (ΔG) is equivalent to -7.1 kcal/mol, and the RMSD equal to 1.951 Å. Thus, the presence of the complex formed by the presence of the ligand 6-deoxycyclitoriacetal (Figure **3B**) (DCTA-Blue), inserted in the characteristic protein of the bee *A. melifera*, was analyzed, where it is observed the position in which it is complexed in the three-dimensional region of the protein (Figure **3B**).

Based on the results available through the simulation, the 2D interaction map was produced, which it aims at the interactions carried out by the ligand, with the amino acid and residues present in the protein. In figure (**3B**), the two-dimensional conformation interaction map is observed, where, it is shown which amino acids the compound 6-deoxyclitoriacetal interacted directly, thus, the ligand has interactions with GLU A:54 with hydrogen bonding type and Pi-Anion type; SER A:57 has two bonds formed of hydrogen bonding type and ASP B:76 with a hydrogen and carbon interaction.

After the analysis between the structural positions performed by the ligands, 6-deoxyclitoriacetal and the clitoriacetal, complexed in the protein structure, it is noted that the structural position of the ligands remains similar, even with the difference in the structure of both, but, present similarities when complexing. Above all, because they perform similar interactions with SER A:57.

Table 1 shows the values of all the existing distances of the 6-deoxyclitoriacetal and clitoriacetal ligands from the reference amino acids present in the characteristic protein of the pheromones of the bee *A. melifera*. Where, in its majority is presented high distances, the largest distance is of the ligand clitoriacetal with the amino acid LYS: B 17 with the value of 21.304 Å and the smallest distance is between the 6-deoxycyclitoriacetal with the amino acid ILE: B 119 with its distance equivalent to 11.024 Å.

With the acquisition of the distance values of the ligands 6-deoxycycloacetal and clitoriacetal it is observable that they remained at a high distance from the reference amino acids, but remained in a similar structural position, so we can deduce that in a three-dimensional position, both compounds complexed to a common region of the protein.

Table 1. Values of the distances between the ligand and the amino acids of the *A. melifera* protein.

	Distances (Å)	
Amino acids	Clitoriacetal	6-deoxyclitoriacetal
VAL A:13	12.640	15.474
VAL B:13	15.218	14.393
ILE A:119	12.475	11.024
ILE B:119	14.019	13.375
LYS A:17	11.708	13.146
LYS B:17	21.304	19.778

CONCLUSION

The development of natural pesticides builds new paradigms in monoculture areas, as they can contribute to their synthesis process, biosafety, and bioactivities. However, it is still necessary to evaluate harmfulness to bees, that is, the main pollinating agents of nature.

Therefore, the natural pesticides 6-deoxyclitoriacetal and clitoriacetal present risks of oral toxicity due to their molecular structure with several hydrogen acceptor sites. However, the toxicological response at the acute level can be immediate, that is, it can cause disturbance, narcosis, or reduction of interactions between bees, and not necessarily death, as they are extremely sensitive to any chemical.

In its validation aspect, the study based between the ligands 6-deoxyclitoriacetal and clitoriacetal with the characteristic protein of the pheromones of the bee *A. melifera* pointed out that both ligands complexed in a region of the protein in which they do not interact directly with the reference amino acids, presenting the smallest distance found the value of 11. 024Å between the ligand 6-deoxycytoriacetal and the amino acid ILE A:119, being this distance is extremely high, so we can deduce that the ligands worked, possibly do not interact with the reference amino acids of the protein of *A. melifera*, consequently does not present toxic potential for these pollinating agents.

It is worth noting that toxicity may vary according to the type of exposure, time, duration, and frequency, as well as the metabolic and daily reactions in which these organisms are exposed to natural pesticides, consequently altering their molecular and biochemical responses. In short, the study is initial and in silico, work is needed to promote in vivo trials and improvements of the method applied to aim at the population welfare of bees and especially the environment.

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