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Sinensetin Contents of Purple and White Purple Variety of *Orthosiphon aristatus* (Blume) Miq

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ABSTRACT:

Context: The cat's whiskers (*Orthosiphon aristatus* (Blume) Miq) plant has been widely used for traditional medicine both in preventive measures and companion therapy. The color of the *O. aristatus* flower is divided into purple, white-purple, and white varieties. To ensure the quality of the *O. aristatus* it is necessary to make standardization efforts. Specific standardization parameters are the identification and determination of marker compound levels. Sinensetin is a marker compound in *O. aristatus*. The research aimed to analyze qualitatively and quantitatively the content of sinensetin in the acetone extract of the purple and white-purple varieties of *O. aristatus* Extraction was carried out using maceration, which began with determining the ratio of the number of leaves and stem simplicia of two varieties of *O. aristatus* and acetone solvent. Sinensetin compound identification was carried out on acetone extract and subfraction. Determination of the levels of sinensetin in acetone extract was carried out by thin-layer densitometry chromatography (TLC-Densitometry). Based on the optimization results using TLC, the weight ratio of the simplicia and the amount of solvent is 1 g of simplicia in 15 mL of solvent. Sinensetin compounds were detected in the leaves' acetone extract and stem of two varieties of *O. aristatus*. The highest sinensetin content was in the acetone extract of purple variety leaves with levels of 0.51% w/w. The acetone extract of the purple variety leaves was further separated by vacuum liquid chromatography and preparative thin-layer chromatography. The results showed a sinensetin compound in the acetone sub-fraction. This study's results can be the basis for the specific characterization of *O. aristatus* especially those grown in Indonesia, to ensure product quality consistency from traditional medicinal plants.

Keywords: *O. aristatus,* purple and white-purple varieties, standardization, sinensetin, qualitative and quantitative analysis.

1. Introduction

O. aristatus is one of the medicinal plants which, based on the results of the research, has various pharmacological activities, including antiviral (Ripim et al., 2018; Faramayuda et al., 2021[a]), prevention and treatment of cancer (Pauzi et al., 2018 rheumatoid treatment and osteoarthritis arthritis (Adawiyah et al., 2018), treating cardiovascular disorders (Abraika et al., 2012), Anti – epilepsy (Kar et al., 2012), enhancing memory (George et al., 2015), antioxidants (Alshawsh et al., 2012; antidiabetic (Mohamed et al., 2011; Mohamed et al., 2010), antiobesity (Yam et al., 2009), treatment of overcome gastric disorders (Yuniarto, et al., 2017). Some studies also report that *O. aristatus* have passed clinical trials (Adnyana et al., 2013; Premgamone et al., 2001). Safety testing of *O. aristatus* extracts in male rats that all animals survived and showed no signs of toxicity. (Muhammad Husin et al., 2001).

Some of the active secondary metabolites of *O. aristatus* are sinensetin, eupatorin and rosmarinate acid (Faramayuda et al., 2021[b]),, and danshensu (Nuengchamnong et al., 2011). *O. aristatus* that grow in Indonesia are classified into three varieties, namely purple, intermediate (white-purple), and white (Faramayuda et al., 2021[c]). The difference between the three varieties can be seen from the morphology of the flower (Faramayuda et al., 2021[d]).

Sinensetin is included in the class of flavonoid compounds, and if they are classified, more specifically are polymethoxy compounds produced by secretory tissue and stored inside or outside the oil glands in plants. Flavone polymethoxy compounds have several pharmacological activities and are part of the plant chemical defense mechanism (Berim and Gang, 2016). The results of a study conducted by Hossain and Ismail in 2016 reported that the level of sinensetin in acetone: water (70:30) extract was 0.32% higher than other solvents (Hossain and Ismail, 2016).

The *O. aristatus* plant has been widely used as raw material for traditional medicine, especially in Indonesia, but many people and industry practitioners use this plant without paying attention to the varieties used. According to Faramayuda, in 2021[b], the methanol extract of the purple variety *O. aristatus* had higher levels of sinensetin

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compared to other varieties. Qualitative and quantitative analysis of sinensetin compounds from acetone extract of two varieties of *O. aristatus* that grow in Indonesia has never been reported. This research can be a reference basis for the specific standardization parameters of the acetone extract of two varieties of *O. aristatus*. Standardization of raw materials is necessary to ensure consistency in the quality of traditional medicinal products.

2. Material and Methods

2.1. Chemicals and reagents:

The chemicals used have the quality of use analysis (pro analysis, p.a), sinensetin (Sigma®), ethyl acetate (Merck®), chloroform (Merck®), acetone (Merck®), silica gel plate pre-coating 60 F254 (Merck®), aqua dest, and silica gel 60 GF254 (Merck®).

Instrumentation:

Glass tools are commonly used in laboratories, macerators, analytical scales (Shimadzu), chambers, ovens (memert), rotary evaporators (Heidolph), vacuum liquid chromatography, and TLC-Densitometry (Camag, Switzerland).

Collection O. aristatus plants

The purple and white-purple varieties of leaves and stems of *O. aristatus* were collected from the Manoko experimental garden, Lembang, West Bandung, Indonesia. The plants were identified at the School of Life Science and Technology, Bandung Institute of Technology. With letter number 6115 / I1.CO2.2 / PL / 2019 .

2.2. Preparation of Extraction

The two varieties of leaves and stems of *O. aristatus* were weighed 1 g each and then added 5 mL of acetone, then let stand for 24 hours. The second day the TLC filtrate I profile was observed, and then the residue was added again with 2.5 mL of acetone. On the third day, the TLC filtrate II profile was observed, and then the residue was added again with 2.5 mL of acetone. Day Four was observed profile of TLC filtrate III. The mobile phase used in TLC observation is chloroform: ethyl acetate 60:40 (Figure. 1).

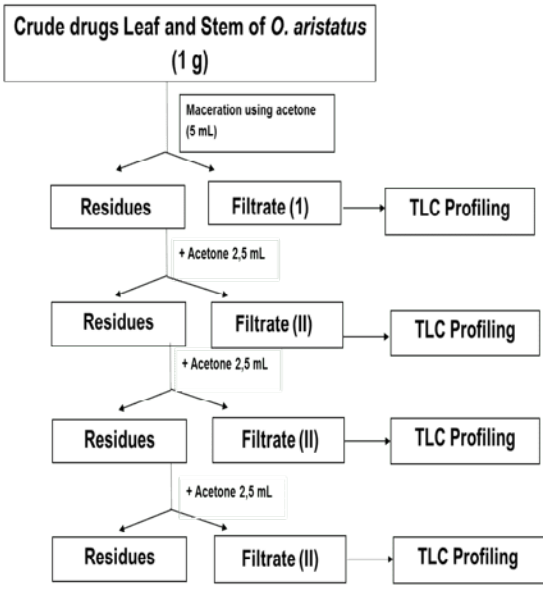

Figure 1. Extraction optimization scheme with maceration

2.3. Extraction of Plant Material

The solvents used to extract plant material are acetone. 100 g of leaves and stems of *O. aristatus* purple variety transferred to macerators, and each added with 1,5 L acetone solvent then macerated for 24 hours. The filtrate was collected, then evaporated using a *rotary evaporator* and concentrated using water baths to form a thick extract.

2.4. Quantitative Analysis

Preparation of standard and sample solutions

Stock solutions of 1 mg / mL of sinensetin in methanol were prepared. The stock solution was diluted with methanol at five concentrations ranging from 60 to 100 µg/mL to create standard solutions. Extracts of acetone from two types of *O. aristatus* were prepared by dissolving 15 mg of each extract in 1 mL of methanol for 45 min as a sample solution.

Instrumentation

Monitoring was carried out in the CAMAG analyzer at a wavelength of 365 nm. Data analysis was carried out using the WinCATS app.

Chromatographic conditions

Standards and samples were applied to a TLC plate with a height of 10 cm and a length of 20 cm. Application volume of 5 mL was performed at a distance of 1 cm from the TLC plate's edge. Toluene mobile phase: ethyl acetate: formic acid: water (3: 3: 1: 0,2) was pre-saturated in the chamber. Observed at a wavelength of 365 nm with densitometry.

2.5. Further separation of the purple variety of O. aristatus acetone extract

Vacuum Liquid Chromatography

The acetone extract was separated by vacuum liquid chromatography using silica gel 60 stationary phases, and 5 g extract was used. The mobile phase used is chloroform: ethyl acetate (60:40) with an isocratic system. The extract was crushed with a small amount of silica gel 60. It then placed it at the top of the column, elution with a mobile phase. Subfraction solutions coming out of the column are collected by volume, which is held every 60 mL. A thin layer chromatography profile was monitored in UV light of 366 nm to the subfraction.

TLC-Preparative and sinensetin Identification

The stationary phase is prepared by mixing 25 grams of silica gel 60 F254 with 50 mL aqua dest (1: 2), shaking in Erlenmeyer (± 90 seconds) until homogeneous. The stationary phase is then poured and flattened on a glass plate measuring 20x20 cm, then allowed to stand for 24 hours in the oven at 100ºC for 30-60 minutes. The mobile phase of chloroform and ethyl acetate 60:40 (Hossain and Ismail, 2016) is put into the chamber and left for 60 minutes, then the subfraction specks on the silica plate. In the elution stage, a plate containing the sample is put into the chamber. The band formed is scraped and dissolved by the mobile phase. The filtrate was evaporated, and monitored the purity of the isolates with TLC.

Analysis of data

Determine the levels of the sinensetin using TLC-Densitometry. All test samples were prepared in three replications. The data are expressed as a mean ± SD. Data processing was carried out by one-way ANOVA, followed

by a multiple-range test by Duncan using SPSS 22 software. P values <0.05 have been considered statistically significant.

3. Result

3.1. Optimazation extraction

Based on the results of the determination of two plant samples identified *O. aristatus* purple flowers and *O.aristatus* white-purple flowers. The optimization of sinensetin extraction with monitoring parameters of the TLC profile showed that on the fourth day, fluorescence from sinensetin was fading on acetone extract of leaves and stems of two varieties of *O. aristatus*. The dry matter ratio used with solvents to maximize sinensetin withdrawal is 1 g: 15 mL (figure 2).

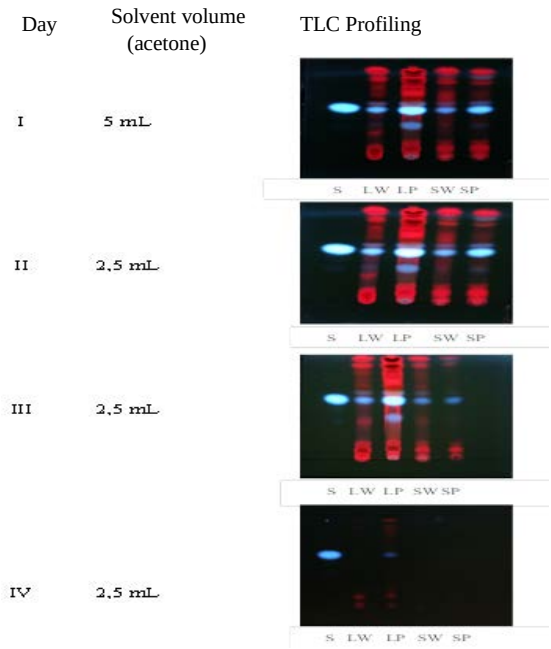

Figure 2. Extraction optimization with TLC profile parameter. Stationary phase silica gel 60 F254, mobile phase chloroform: ethyl acetate (60:40), Rf sinensetin : 0.61. S: sinensetin, LW: leaf extract white-purple., LP: leaf extract purple, SW: stem extract white-purple, SP: stem extract purple.

3.2. Quantitative Analysis

The analysis of sinensetin levels in the acetone extract of the *O. aristatus* leaves and stems used densitometric instruments. The regression equation of the variation in sinensetin's standard concentration is y = 86.872x - 4438.5 with an r^2 value of 0.9954. Three repetitions were carried out for each sample, and the sinensetin compound was detected at Rf 0.57 (figure 3).

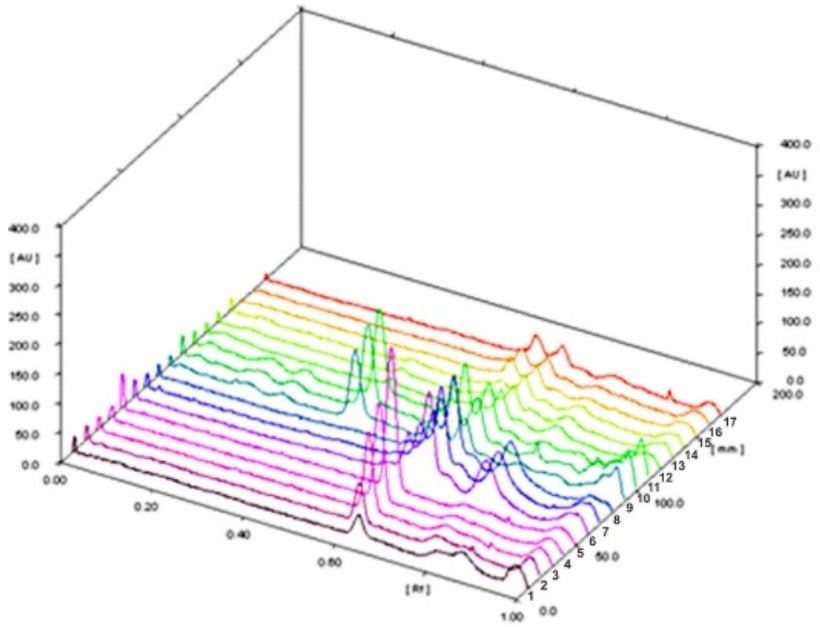

Figure 3. 3D-TLC chromatogram of the acetone extract of two varieties *O. aristatus* and the standard sinensetin. Track 1: sinensetin 60 µg / mL, 2: sinensetin 70 µg / mL, 3: sinensetin 80 µg / mL, 4: sinensetin 90 µg / mL, 5: sinensetin 100 µg / mL, 6-8: purple variety leaves acetone extract (3 replications), 9-11: white-purple variety leaves acetone extract (3 times replication), 12-14: purple variety stem acetone extract (3 replications), 15-17: white-purple variety stem acetone extract (3 times replication).

Table 1. Levels Determination of sinensetin acetone extract of two varieties *O. aristatus* with TLC-densitometry

Sample	Sinensetin (% w / w) ± SD (n = 3)
purple variety leaves acetone extract	0.51 ± 0.01[a]
white-purple variety leaves acetone extract	0.48 ± 0.01[a]
purple variety stem acetone extract	0.43± 0.01[b]
white-purple variety stem acetone extract	0.39 ± 0.02[b]

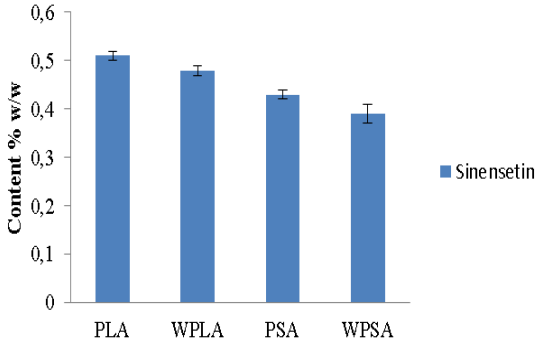

Figure 4. Comparison sinensetin levels of acetone extracts of two varieties of *O. aristatus*. PLA = purple variety leaves acetone extract, WPLA = white-purple variety leaves acetone extract, PSA = purple variety stem acetone extract, WPSA = white-purple variety stem acetone extract

3.3. Further separation of the purple variety of O. aristatus acetone extract

In the separation stage using vacuum liquid chromatography using a mobile phase of chloroform: ethyl acetate 60: 40 with an isocratic system, eight subfractions were obtained. Sub-faction 1-5 contains a sinensetin compound (Rf 0.45), but what is continued to the separation stage is sub-faction 5 (SF5) because there is not too much chlorophyll, so it is easier to isolate (Figure 5). Further separation of SF5 was using preparative thin-layer chromatography (PTLC) with the mobile phase of chloroform: ethyl acetate 60: 40 obtained two bands. The second band is separated by scraping and then dissolved in a mobile phase to filter further and identify the filtrate (figure 6).

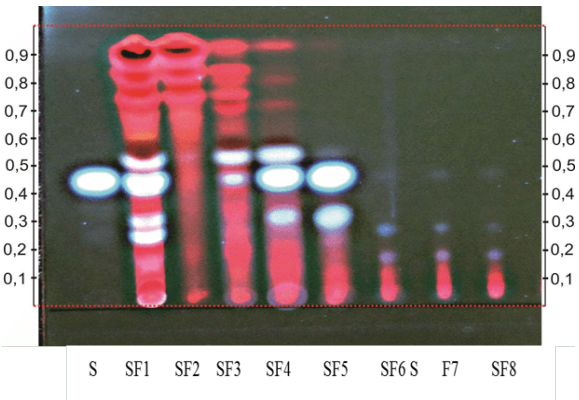

Figure 5. TLC Profile Sub Fraction 1-8 of *O. aristatus*. Stationary phase silica gel 60 F254, mobil phase = chloroform : ethyl acetate 60 : 40. S: Sinensetin, SF1 = sub-faction 1, SF2 = sub-faction 2, SF3 = sub-faction 3, SF4 = sub-faction 4, SF5 = sub-faction 5, SF6 = sub-faction 6, SF7 = sub-faction 7, SF8 = sub-faction 8.

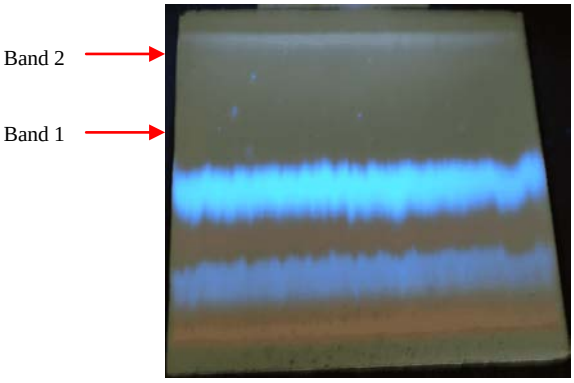

Figure 6. TLC Preparative Profile of subfraction 5. Stationary phase silica gel 60 F254, mobile phase chloroform: ethyl acetate 60: 40

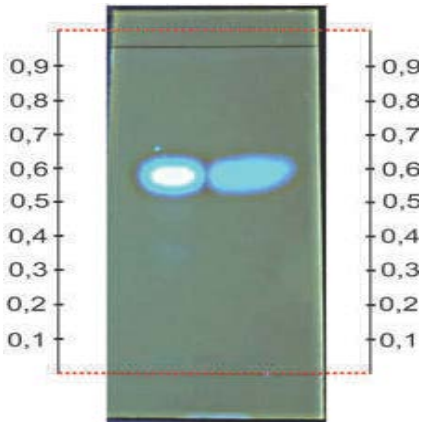

Figure 7. TLC Profile of isolate. Stationary phase silica gel 60 F254; mobile phase chloroform: ethyl acetate 60: 40. S: Sinensertin and I: Isolate

Band 2 filtrate was identified using TLC and Sinensetin standard; the results showed the same Rf between isolate and standard (figure 7).

4. Discussion

4.1. Optimazation extraction

From monitoring the TLC profile, the fluorescence of the sinensetin purple variety is brighter than the white-purple variety; this shows that the purple variety level is higher than that of the white-purple variety. Earlier research reports that the purple variety of sinensetin levels were higher than white varieties (Lee, 2004). According to research conducted by Faramayuda in 2021, it was reported that the population of purple varieties of cat whiskers is decreasing (Faramayuda et al., 2021[e]). Therefore, it is necessary to propagate plants, one of which is the plant tissue culture method. Several studies have reported that the propagation technique with modified in vitro culture has succeeded in producing seeds of Phalaenopsis amabilis (L.) Blume (Mose et al., 2020), Artemisia herba-alba (Shibli et al., 2017), Chrysanthemum morifolium (Shatnawi et al. al., 2010).

4.2. Quantitative Analysis

The sinensetin compound's content in the acetone extract of the purple variety *O. aristatus* was 0.51% w/w, while the white-purple variety was 0.48% w/w. Statistically, there was no significant difference. Two varieties of sinensetin cat whiskers were detected with

levels of 0.43% w / w on the purple variety and 0.39% w/w on the white-purple variety (table 1; figure 4). Sinensetin levels in purple varieties were more significant than those in white-purple varieties. This result was in line with those reported by Febjlasmi in 2018, where the levels of sinensetin from the purple variety *O. aristatus* were greater than the white and white-purple varieties. The levels of sinensetin in the acetone extract of the purple variety cat whiskers were more significant than that reported by previous researchers, where the sinensetin content of the acetone: water extract (70:30) cat whiskers was 0.32% w / w and methanol: water (1; 1) 0.15. % w / w (Hossain et al., 2016). Cai (2018) explains that the levels of sinensetin in the 50% ethanol extract of cat whiskers are 0.27% w / w and in the stem is 0.01% w / w. The average concentration of the sinensetin compound in the purple variety cat whiskers extract that grows at 18 locations in Indonesia is 0.043% w / w. Methanol extract: water (50: 50) of cat whiskers that grow in Malaysia is 0.0057% w / w (Guo et al., 2019). Sinensetin content of 70% ethanol extract of purple variety cat whiskers is 0.0182% w / w (Batubara et al, 2020). Sinensetin, which is lipophilic, is more stable in low polar solvents such as isopropanol, chloroform, and acetone (pang and gimbung, 2014). Based on the chemical structure, sinensetin is a polymethoxy (pentamethoxyflavone) flavone group, the methoxy group substitution at positions 5, 6, 7, 3 '4' (Han Jie et al., 2021). The presence of this methoxy group makes sinensetin less polar.

4.3. Further separation of the purple variety of O. aristatus acetone extract

In the Further separation, the plant used is purple variety leaves. The acetone solvent selection in sinensetin isolation was based on Hossain's research, and Ismail in 2016 reported that the acetone extract: water has higher sinensetin levels than other extracts (Hossain and Ismail, 2016). The yield of acetone extract is 18.5% w / w. The isolates suspected of being sinensetin were obtained as much as 1.8 mg from 100 gr of simplicia *O. aristatus* purple varieties. The optimization of sinensetin separation by VLC will be better if the elution were performed by gradient elution with n-hexane-ethyl acetate. In this research the solvent used for VLC was too polar. Several studies on the isolation and identification of sinensetin have been reported, including sinensetin obtained from the ethyl acetate fraction of *O. aristatus*, which was separated using column chromatography and then eluted with 100% hexane, followed by up to 100% hexane: ethyl acetate and up to 100% ethyl acetate: methanol (Samidurai et al., 2019). The methanol extract of the leaves was extracted with-hexane, chloroform, ethyl acetate, and butanol. The chloroform fraction was purified, and six compounds were obtained, one of which is sinensetin (Hossain and Mizanur Rahman, 2015). Two methoxy flavonoids were isolated and identified from the leaves of *Orthosiphon stamineus*, Benth, which are known as sinensetin and flavones 5,7,8,4' tetra methoxy, extraction with Soxhlet using ethyl acetate as a solvent followed by liquid-liquid extraction with $CuCl_2$ and NaOH to reduce the effect chlorophyll content in the isolation process (Febriani et al.,2017). The HPLC method was developed for the separation and determination of three methoxylated flavones: sinensetin, eupatorium, and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone in *Orthosiphon stamineus*. All compounds were separated using reverse phase C18, Lichrosorb column with methanol-water mobile phase -tetrahydrofuran 45: 50: 5 v / v (Akowuah, et al., 2004). This study provides additional information regarding the composition of the number of simplicia and solvents, which can then be developed for the basis of production or optimization of the isolation of sinensetin from *O. aristatus*. Several previous studies reported attempts to isolate sinensetin, including 4 kg of simplicia of the cat's whiskers producing 75 mg of sinensetin (Tezuka et al., 2000). Isolation of sinensetin from 500 g of *O. aristatus* using an LH-20 Sephadex column resulted in an isolate of 3.03 mg (Yuliana et al., 2009). Hossain and Mizanur Rahman (2015) reported that from 1 kg of plant material, *O. aristatus* produced 2.6 mg of sinensetin isolate.

5. Conclusion

The acetone extract of the leaves and stem of the purple variety *O. aristatus* had higher sinensetin levels than the white-purple variety.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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