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Phytochemical Screening and Cytotoxic Activity in *Chromolaena odorata* (L.) R.M. King & H. Rob. Leaf and Root Extracts by Brine Shrimp Lethality Assay

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Abstract

Chromolaena odorata (L.) R.M. King & H. Rob., a rapidly expanding invasive plant, is traditionally used in ethnomedicine for wound healing, antibacterial, and anti-inflammatory purposes. This study analyzed the methanolic and aqueous extracts of the leaf and root of C. odorata qualitatively for major secondary metabolites and tested for cytotoxicity against Artemia salina nauplii using the Brine Shrimp Lethality Assay (BSLA). Phytochemical screening results showed that methanol and aqueous extracts comprised tannins, terpenoids, alkaloids, flavonoids, and saponins. The aqueous extract of roots showed the maximum cytotoxic activity on the Brine Shrimp nauplii with Lethal Concentration 50 (LC_{50})=62.61 µg/mL, indicating the presence of potential bioactive or cytotoxic compounds. Furthermore, it was found that root extracts were more effective at cytotoxicity than leaf extracts and that both leaf and root aqueous extracts were more cytotoxic to nauplii than the methanolic extracts. The findings suggest that C. odorata contains bioactive compounds with potential cytotoxic activities, warranting further investigation for anticancer activities and identification and characterization of the bioactive compounds.

Keywords: Aqueous extract, Bioactive, Invasive, LC_{50}, Methanolic.

Introduction

Chromolaena odorata (L.) R.M. King & H. Rob. (Commonly known as Siam weed) belongs to the family Asteraceae, which is a perennial shrub known for its ethnomedicinal value across tropical and subtropical regions (Iwu, 1993). It is a native plant of Central and North America (Nesom, 2006), and has been spread to various regions of the world, including Australia, Africa, and Asia as an invasive alien species (Chen et al., 2011). In the case of Nepal, it grows in tropical and subtropical parts, mostly in the eastern and central parts. Traditionally, *C. odorata* has been valued for its various curative properties regardless of its reputation as an invasive weed. It is used by local healthcare providers to treat injuries, fungal infections, pounding head, odontalgia, diarrhea, stomach issues, coughs, and skin diseases (Omokhua et al., 2017). It possesses parasiticidal, antioxidant, pain-relieving, febrifuge, spasmolytic, inflammation-reducing, antibacterial, antimalarial, anti-tumor, antihyperglycemic, and wound-healing activities (Phan et al., 1998; Omokhua et al., 2016; Akinwunmi et al., 2017). Among its many therapeutic advantages, this plant's wound-healing capabilities are thought to be

especially interesting. On the other hand, because of its adverse impacts on the agricultural sector, ecosystems, and lives, *C. odorata* has become a financial burden.
The chemicals found naturally in plants are called phytochemicals, and the process of identifying the different groups of phytochemicals in different plant parts is known as phytochemical screening (Wachira et al., 2024). Phytochemical screening serves as a preliminary step to determine whether potentially therapeutic or bioactive compounds are present in the plant. It helps prioritize plants for further, more detailed studies, including quantitative analysis, isolation, or bioassays. Qualitative tests are usually simple, fast, and inexpensive, making them ideal for use in field or resource-limited settings. Among the many different types of chemical compounds found in plants are alkaloids, terpenoids, phenols, flavonoids, tannins, lignans, coumarins, and anthraquinones. These are bioactive substances synthesized in plants by primary and secondary metabolic pathways that benefit human health by curing a variety of illnesses and safeguarding the plant from infections, diseases, or predation by microorganisms, pests, diseases, or predators. Plants have long been used as sources of therapeutic agents due to their diverse bioactive compounds. It has demonstrated antibacterial, antioxidant, and anti-inflammatory properties (Akinmoladun et al., 2007). Despite its invasiveness, it has many phytochemicals that are worth further research. Phytochemical studies have identified flavonoids, alkaloids, terpenoids, phenolic compounds, tannins, coumarins, steroids, and saponins as major bioactive constituents (Nworgu et al., 2022; Budha Magar et al., 2023; Uwejigho et al., 2025). The leaves, roots, and flowers of *C. odorata* contain a variety of bioactive compounds, including phenolic acids (protocatechuic acid, ferulic acid, and vanillic acid), mixtures of flavonoid aglycones (sinensetin, rhamnetin, tamarixetin, and kaempferide) (Phan et al., 2001), essential oils (himachalol, 7-isopropyl-1,4-dimethyl-2-azulenol, androencecalinol, and 2-methoxy-6-(1-methoxy-2-propenyl) naphthalene) (Joshi, 2013), and various other chalcones (Suksamrarn et al., 2004).

The ability of a test substance (such as plant extracts or synthetic compounds) to cause mortality in *Artemia salina* (brine shrimp) larvae (nauplii) is known as cytotoxicity in the brine shrimp lethality assay (BSLA) (Carballo et al., 2002; Banti and Hadjikakou, 2021). It is a simple, cost-effective method for assessing cytotoxicity in crude plant extracts (Meyer et al., 1982). BSLA is a quick and affordable way to check for substances that are poisonous or bioactive and may be cytotoxic to higher organisms or human cell lines. This study is necessary for the scientific validation of the traditional medicinal uses of *C. odorata* by identifying major phytochemicals that may be responsible for therapeutic effects. The cytotoxic effects of *C. odorata* extracts may indicate possible anticancer or antiproliferative and pesticidal compounds, providing a foundation for further pharmacological studies. Thus, it can contribute to drug discovery efforts by exploring plant-derived cytotoxic agents. This study aims to identify major groups of phytochemicals in aqueous and methanolic extracts of roots and leaves of *C. odorata* as a preliminary phytochemical screening, and evaluate their cytotoxic activities in Brine shrimp nauplii or larvae using BSLA.

Materials and methods

Collection of plants and authentication

Fresh leaves and roots of *C. odorata* were collected in August 2024 from the Butwal area of Rupandehi district, Nepal (Fig. 1). The plant specimen was authenticated by tallying the herbarium specimens of the Department of Botany, Butwal Multiple Campus, Tribhuvan University, and a voucher specimen (Voucher no. 012B) was deposited in the Department of Botany.

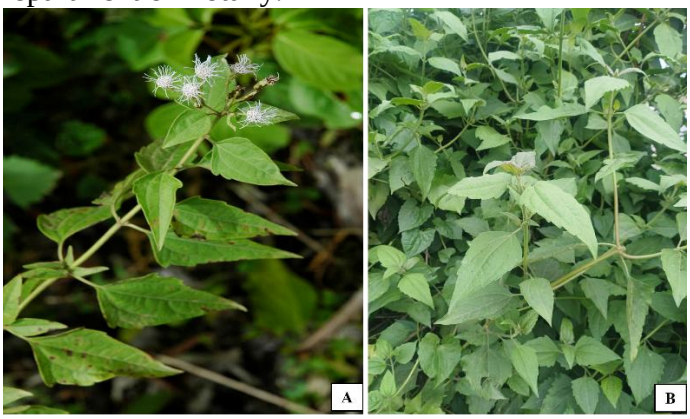

Figure 1. *Chromolaena odorata*: (A) *C. odorata* at the flowering stage, (B) *C. odorata* at the vegetative stage

Preparation of extracts

The collected plant material was cleaned with water, shade-dried, and ground into fine powder. Methanolic and aqueous extracts were prepared. For methanolic extract preparation, 100 g of leaf and root powder were cold macerated separately (at room temperature of 25^{o}C) in 500 mL of 95% methanol for 72 hours with occasional shaking and kept in the water bath at 40^{o}C for one and a half hours. Then, the mixture was filtered. This process was repeated three times, and the filtrate was evaporated using a water bath at 37^{o}C. For aqueous extract preparation, 100 g of leaf and root powder were cold macerated in 500 mL distilled water for 72 hours, and then hot (at 50^{o}C) macerated for one and a half hours. The entire mixture was filtered and then concentrated in a water bath at 60^{o}C. The extracts were stored in vials at 4^{o}C in the refrigerator for further use.

Phytochemical screening

Aqueous and methanol extracts of leaves and roots were tested using standard qualitative tests for different types of secondary metabolites, including alkaloids, flavonoids, tannins, saponins, terpenoids, and glycosides, following Harborne (1998), Sofowora (1993), and Trease and Evans (1989).

Alkaloid test

Meyer's Test: The extract of the plant (2 mL) was mixed with 2 mL of 2% (v/v) HCl. Meyer's reagent, which is a solution of potassium mercuric iodide, was then added in little drops. Alkaloids are present when a white, creamy precipitate forms upon shaking.

Tannin test

Tannins are polyphenolic compounds that form colored complexes with ferric ions (Fe^{3+}), making this test a standard for qualitative identification. The extract of the plant (2 mL) was combined with 2 mL of 5% (v/v) $FeCl_3$. The formation of blue, black, green, or brown color complexes shows the presence of tannin in the extract.

Flavonoid test

Flavonoids are polyphenolic compounds with antioxidant properties, and their presence in plants can be confirmed through specific color reactions.

Shinoda Test: Small pieces of magnesium ribbons were put into a test tube containing 2-3 mL of plant extract. After adding 1-2 mL of diluted HCl, the mixture was slowly heated in a water bath for 2-3 minutes before being allowed to cool. Flavonoids are confirmed when a red, pink, magenta, or orange-red color forms.

Alkaline Reagent Test: In a test tube, 1-2 mL of a 5–10% NaOH solution was combined with 2–3 mL of the plant extract. Flavonoids are indicated by a yellow appearance or by an intense yellow coloring. Following acidification with 1-2 mL of HCl, the color turns colorless.

Glycosides test

Liebermann's Test: This test is used to detect **glycosides**, particularly those with steroidal or terpenoid aglycones. In a test tube, 2 mL of the plant extract and 2-3 mL of acetic acid were combined. A clear lower layer was produced by adding 1-2 mL of concentrated sulfuric acid along the test tube's wall. A blue-green ring appears where the two layers meet when a glycoside is present.

Keller-Kiliani Test: The extract of the plant (2 mL) was mixed with 2 mL of acetic acid containing 1–2 drops of 5% $FeCl_3$. It was mixed well and gently warmed in a water bath for 1–2 minutes to hydrolyze the glycoside. Then, 1–2 mL of concentrated H_2SO_4 was added along the side of the test tube to form a distinct lower layer. The formation of a **brown ring** at the interface indicates the presence of cardiac glycosides.

Terpenoids test

Salkowski Test: The extract of the plant **(**2–3 mL**)** was mixed with 1–2 mL of chloroform in a test tube. It was shaken gently to ensure homogeneity. Concentrated H_2SO_4 (1-2 mL) was added along the side of the test tube to form a distinct lower acidic layer. The formation of a reddish-brown or yellow-brown ring at the junction shows the presence of terpenoids.

Saponin Test

Froth Test: **Plant extract** (1 mL) was mixed with 10 mL of distilled water in the test tube and shaken the mixture vigorously for about 30 seconds to 1 minute. The mixture was allowed to stand undisturbed for 10–15 minutes. Persistent froth (foam) of height 1 cm or more that remains for 10 minutes or longer indicates the presence of saponins.

Steroid test

Salkowski Test: Plant extract (1-2 mL) was mixed with 2-3 mL of chloroform in a test tube, and an equal volume of H_2SO_4 was added. A red or brown ring at the interface confirms steroids on shaking.

Carbohydrate test:

Test for reducing sugar (Benedict's Test): The extract of the plant (1 mL) was mixed with **2 mL of Benedict's reagent** in a test tube, and it was boiled in the water bath for 5-10 minutes. The production of the orange-red precipitate indicates the presence of reducing sugar.

Test for reducing sugar (Fehling's Test): Plant extract (1 mL) was mixed with an equal volume of Fehling's solution A and B in a test tube and boiled for 5 minutes. The production of a brick-red precipitate indicates the presence of reducing sugar.

Phenolic test

Ferric Chloride Test: Plant extract (1-2 mL) was mixed with 1–2 drops of 5% $FeCl_3$ solution. A blue, green, or black color indicates phenolic acids.

Brine Shrimp lethality assay

It was performed using the protocol described by Meyer et al. (1982) and Fatope et al. (1993).

Hatching of Brine Shrimp

An artificial seawater (salt water) was prepared using 3.5 g NaCl in 100 mL of distilled water. In a beaker filled with artificial seawater (pH 8.0), brine shrimp (*Artemia salina*) eggs were hatched under constant aeration and illumination (60-watt bulb) for 48 hours at 23^{o}C.

Cytotoxicity test

Extracts (1 mg/mL) were dissolved in DMSO and diluted to various concentrations (50, 120, 250, 500, 1000 µg/mL) with salt water. Each concentration was tested in triplicate using 10 nauplii per test tube containing 1 mL of salt water. For the negative control, 10 nauplii were added to saltwater without extracts. For positive control, potassium dichromate ($K_2Cr_2O_7$) was used instead of extracts. After 24 hours of incubation at room temperature, the number of surviving nauplii was counted, and LC_{50} (half-lethality concentrations) values were calculated using regression analysis.

$$Percentage\ mortality\ Nauplii = \frac{No.of\ dead\ Nauplii}{No.of\ live\ Nauplii\ taken} \times 100$$

Data analysis

The average number of surviving nauplii and percentage mortality of nauplii were calculated in Microsoft Excel 2010. Similarly, half-lethality concentrations (LC_{50}) of various extracts to Brine shrimp nauplii were calculated using linear regression in Microsoft Excel 2010.

Results and discussion

Percentage of extract yield

This study showed that the highest percentage of extract yield (21.45%) was found in the aqueous extract of *C. odorata* leaf than in other extracts (Table 1). This showed that water yielded more extracts than methanol. This result was supported by the findings of other researchers in C. odorata (Hanphakphoom & Krajangsang, 2016; Oko et al., 2017). It might be because it is more polar than methanol, which dissolves a wider variety of hydrophilic substances and aids in the better swelling and breakdown of plant matter. However, the percentage of extract yield depends on the types of solvent used (polar or nonpolar), plant parts, particle size, extraction methods, extraction run time, etc.

Table 1. Percentage of extract yield in leaf and root powder of *C. odorata* in various solvents.

S.N.	Plant parts	Solvent used	Methods of extract preparation	% Extract yield
1.	Leaf	Methanol (95%)	Cold and hot maceration	12.12
		Aqueous	Cold and hot maceration	21.45
2.	Root	Methanol (95%)	Cold and hot maceration	8.34
		Aqueous	Cold and hot maceration	14.54

Phytochemical constituents

All plants produce primary metabolites such as carbohydrates, proteins, lipids, and nucleic acids.

Testing for carbohydrates is not primarily about identifying a drug compound. Instead, its significance lies in understanding bioavailability and recognizing the direct and indirect roles carbohydrates play in the therapeutic and commercial value of plants. The present study showed the presence of carbohydrate in both methanolic and aqueous extracts of the leaves and roots of *C. odarata* (Table 2). Despite being primary metabolites, glycosidation connections allow carbohydrates to be included in a wide variety of secondary metabolites. Gums and mucilages are made of a polymer of uronic acids and carbohydrates (Asif et al., 2011). Mucilage mostly functions as a local lubricant or relieving agent when it comes into contact directly with skin or mucus membrane surfaces. Gastrointestinal activity is greatly affected by their respective indigestible and hydrophilic characteristics (Anbalahan, 2017), and inflammation of the bowels and throat pain (WHO, 1999). However, **not all plants produce all types** of secondary metabolites, and **some may not produce any** or may produce them in negligible amounts. Often, they serve **ecological functions** like defense (against herbivores and pathogens), attraction (pollinators), or competition (allelopathy). Their presence and type vary **depending on species, habitat, stress conditions**, etc. The present study found that all the extracts of *C. odorata* leaf and root showed alkaloids, flavonoids, tannins, saponins, phenolics, and terpenoids, while glycosides were absent in all extracts (Table 2). Previous research findings, particularly in leaf extracts, supported this finding (Phan et al., 2001; Nworgu et al., 2022; Budha Magar et al., 2023; Uwejigho et al., 2025). A few studies were found in the root extracts of *C. odorata* for the phytochemical analysis. However, phytochemical screening of the leaves and roots revealed that the main groups of compounds were more concentrated in

the roots than in the leaves. This helps to prove that the distribution of bioactive compounds depends on the parts of the plant. Several biological (developmental stage, species, genetics, and plant organ), environmental (light and temperature, soil nutrients, biotic stressors, climate, and altitude), and technical factors (extraction techniques, solvent polarity) affect whether secondary metabolites are found in different kinds of plant extracts (Barthwal & Mahar, 2024; Cao et al., 2025; Esparza-Orozco et al., 2025). The color intensity in the test solution has been used as an approximate indicator for the amount or concentration (low, moderate, and abundant) of the metabolites in different extracts. However, this qualitative test is unable to accurately quantify the secondary metabolites present in various extracts. Based on the color intensity of the tested solution, this investigation demonstrated that the majority of the major metabolites were found to be abundant (+++) in the aqueous extracts of both leaves and roots when compared to methanol extracts (Table 2). The concentration of secondary metabolites in aqueous versus methanol extracts depends on several factors, including the polarity of the compounds, extraction methods, and solvent interactions. Gonzalez-Palma et al. (2016) found that when *Pleurotus ostreatus* was extracted by decoction (boiling) and maceration at room temperature, phenolic compounds such as tannins and flavonoids exhibited higher quantities in aqueous extracts. Similarly, Olivia et al. (2021) reported that because saponins and alkaloids are soluble in water, they accumulate more in aqueous extracts of *Hibiscus asper* leaves.

Table 2: Major phytochemical constituents of *C. odorata* leaf and root extracts.

Phytoconstituents	**Leaf**		**Root**	
	Methanol	**Aqueous**	**Methanol**	**Aqueous**
Alkaloid	(+++)	(+++)	(++)	(+++)
Flavonoid	(+++)	(+++)	(++)	(+++)
Tannin	(++)	(+++)	(++)	(+++)
Saponin	(+)	(++)	(+)	(++)
Terpenoid	(+++)	(++)	(++)	(+++)
Glycoside	(-)	(-)	(-)	(-)
Steroid	(-)	(++)	(+)	(++)
Carbohydrate	(+)	(+)	(+)	(+)
Phenolic acid	(+)	(++)	(+)	(++)

(+) = *present in low amount,* (++) = *moderate,* (+++) = *abundant, (-) = absent*)

Brine Shrimp Lethality Assay

The present study showed that the aqueous extract of the root showed the highest lethality (LC_{50} = 62.61 µg/mL), indicating significant cytotoxicity (Table 6). A lower LC_{50} value indicates greater cytotoxicity to nauplii than a larger LC_{50} value. Potassium dichromate was used as a positive control to compare toxicity; the LC_{50} was 45.5 µg/mL. The LC_{50} for nauplii was found to be less than 1000 µg/mL for all extracts of *C. odorata* (Tables 3 to 6). Meyer et al. (1982) defined a toxic LC_{50} value as one that is less than 1000 µg/mL and a non-toxic value as one that is greater than this threshold. However, Karchesy et al. (2016) indicated cytotoxic activity is poor at LC_{50} of 500-1000 µg/mL, medium at 100-500 µg/mL, and strong at 0-100 µg/mL. According to this

interpretation, aqueous extracts of root (LC_{50}=62.61 µg/mL) and leaf (LC_{50}= 76.3 µg/mL) were highly toxic, the methanol extract of root (LC_{50}=489.69 µg/mL) was moderately toxic, and the methanolic extract of the leaf (LC_{50}=660.56 µg/mL) was weakly toxic. The findings indicated that its cytotoxic effects could be explained by the presence of secondary metabolites, such as flavonoids, tannins, and alkaloids, and the phytochemical screening confirmed their presence in methanolic and aqueous extracts of *C odorata* for various bioactivities.

Compared to methanolic extracts, aqueous extracts of the leaf and root were more cytotoxic to nauplii. It might be because aqueous extracts contain more bioactive compounds than methanolic extracts. Budha Magar et al. (2023) also reported that the aqueous extract of *C. odorata* flower showed higher cytotoxicity to brine shrimp nauplii than the methanolic extract. Moreover, Waghulde et al. (2019) found that the aqueous extracts of *Annona reticulata* leaves and *Allium sativum* bulbs exhibited significantly higher cytotoxicity to brine shrimp nauplii than their alcoholic extract. Similarly, the aqueous extract of *C. odorata* root was more cytotoxic to nauplii than the leaf aqueous extract. Pyrrolizidine alkaloids were identified in *C. odorata* roots by Biller et al. (1994), and Fu et al. (2004) reported that these compounds have genotoxicity, hepatotoxicity, DNA damage, cytotoxicity, or carcinogenic effects on living organisms.

Table 3: Percent mortality of Brine shrimp nauplii and LC_{50} of methanolic leaf extracts of *C. odorata*

Extract Concentration (µg/mL)	**No. of surviving Nauplii after 24 hours**			**Average no. of surviving Nauplii**	**Dead Nauplii**	**% Mortality**	**LC_{50} (µg/mL)**
	N_1	N_2	N_3				
50	8	9	7	8.00	2.00	20.00	660.56
120	8	7	8	7.66	2.33	23.33	
250	7	6	7	6.66	3.33	33.33	
500	6	5	5	5.33	4.66	46.66	
1000	3	4	4	3.66	6.33	63.33	

Table 4: Percent mortality of Brine shrimp nauplii and LC_{50} of aqueous leaf extracts of *C. odorata*

Extract Concentration (µg/mL)	**No. of surviving Nauplii after 24 hours**			**Average no. of surviving Nauplii**	**Dead Nauplii**	**% Mortality**	**LC_{50} (µg/mL)**
	N_1	N_2	N_3				
50	6	5	6	5.66	4.33	43.33	76.3
120	5	5	6	5.33	4.66	46.66	
250	3	4	3	3.33	6.66	66.66	
500	1	2	1	1.33	8.66	86.66	
1000	0	0	0	0.00	10.00	100.00	

Moreover, the LC_{50} value of <100 µg/mL in the aqueous extract falls within the range considered significantly bioactive (Meyer et al., 1982), indicating the potential for anticancer drug development. The methanol extract, although less potent, also exhibited moderate cytotoxicity.

Table 5: Percent mortality of Brine shrimp nauplii and LC_{50} of methanolic root extracts of *C. odorata*

Extract Concentration (µg/mL)	No. of surviving Nauplii after 24 hours			Average no. of surviving Nauplii	Dead Nauplii	% Mortality	LC_{50} (µg/mL)
	N_1	N_2	N_3				
50	8	9	9	8.66	1.33	13.33	489.69
120	8	9	7	8.00	2.00	20.00	
250	6	6	5	5.66	4.33	43.33	
500	4	4	2	3.33	6.66	66.66	
1000	2	3	3	2.66	7.33	73.33	

Table 6: Percent mortality of Brine shrimp nauplii and LC_{50} of aqueous root extracts of *C. odorata*

Extract Concentration (µg/mL)	No. of surviving Nauplii after 24 hours			Average no. of surviving Nauplii	Dead Nauplii	% Mortality	LC_{50} (µg/mL)
	N_1	N_2	N_3				
50	6	5	5	5.33	4.66	46.66	62.61
120	4	5	5	4.66	5.33	53.33	
250	4	5	4	4.33	5.66	56.66	
500	1	2	1	1.33	8.66	86.66	
1000	0	0	0	0.00	10.00	100.00	

Table 6. Comparative LC_{50} values and the toxic levels of *C. odorata* leaf and root extracts.

Plant parts	Extracts	LC_{50} (µg/mL)	Toxicity level
Leaf	Methanol	660.56	Weakly toxic
	Aqueous	76.3	Highly toxic
Root	Methanol	489.69	Moderately toxic
	Aqueous	62.61	Highly toxic
Potassium dichromate (Positive control)		45.5 µg/mL	

Conclusion

The phytochemical screening of *Chromolaena odorata* leaf and root extracts revealed the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, and phenolic compounds. The aqueous extract of the root showed the **strongest cytotoxic activity** against brine shrimp nauplii (LC_{50} = 62.61 µg/mL), followed by the aqueous leaf extract (LC_{50} = 76.3 µg/mL), indicating high toxicity. Methanolic extracts were less cytotoxic. These results suggest that *C. odorata*, especially its root aqueous extract, contains **bioactive compounds with significant cytotoxic potential**, supporting its traditional medicinal use and justifying further investigation for anticancer or pesticidal

applications. Moreover, further *in vitro* anticancer studies, fractionation, and bioassay-guided isolation are recommended for the identification of allelochemicals and bioactive compounds responsible for cytotoxicity.

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Conflict of interest
The authors declare no conflict of interest.

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