Evolution Effectiveness of *Clinacanthus nutans* against liver cancer in White male rats

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Abstract

Background: The carcinogen thiacetamide (TAA) is a potent toxin that damages many tissues through oxidative stress, elevated pro-inflammatory indicators, and apoptosis. On the other hand, Clinacanthus nutans (C. nutans) have illustrated antioxidant and anti-inflammatory features. **Method**: The investigation comprised five sets of rats (n = 6): the sham set (regular diet and water), the vehicle set (water was utilized as a solvent), the TAA set (orally 200 mg/kg for 2 weeks), the C. nutans set (orally 10 mg/kg for 2 weeks) and the combination (C. nutans + TAA) set. Serum specimens were analyzed for cytokine and apoptotic proteins release (TNF α, BCL2, CEA, IL-1β, IL-6, TGF-β, 3 Caspase, and MCP-1) and oxidative and anti-oxidative markers (GSH, MDA, SOD, and COX). In addition, serum specimens were analyzed for liver function markers (ALP, AST, ALT, and T-BIL). **Results**: The findings demonstrated a substantial decrease (p less than 0.05) in GSH, SOD, COX, and BCL2 levels in the TAA set comparison with the C. nutans and combination sets. Conversely, liver enzymes, bilirubin, TNF-α, IL-6, IL-1β, TGF-β, MCP-1, and Caspase 3 levels increased insubstantially (p higher than 0.05) relative to other sets. C. nutans dissolved in water for 2 weeks showed a substantial decrease in liver enzymes and bilirubin, as well as TNF-α, IL-6, and IL-1β, TGF-β, MCP-1, and Caspase 3 levels, and a substantial increase (p less than0.05) in GSH, SOD, COX, and BCL2 levels when comparison with the TAA-treated set. **Conclusion**: C. nutans leaves possess anti-inflammatory and antioxidant features, which can effectively scavenge free radicals and inhibit tumor growth.

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Introduction

An organosulfur fungicide called thioacetamide (TAA) prevents the germination of fungus spores by producing sulfide ions. Furthermore, it is utilized as a substitute for hydrogen sulfide in qualitative inorganic analysis in the chemical and pharmaceutical industries. Humans can be exposed to TAA by ingesting, inhalation, or skin absorption due to its water solubility and toxicity-causing potential (1). Thioacetamide S-oxide is formed after TAA is bioactivated, and this, in turn, causes peroxide radicals to be produced. Reactive oxygen species (ROS) production follows from this (2). It has been established that these ROS initiate oxidation reactions, including lipid peroxidation to unsaturated lipids, or trigger other reactions with sulfhydryl compounds. These processes have the potential to result in liver injury (3).

Clinacanthus nutans (*C. nutans*), or Sabah Snake Grass, is a species of the Acanthaceae family that has therapeutic potential in modern and traditional herbal medicine, involving antiviral (7), antimicrobial (6), anti-inflammatory (5), anti-diabetic (4), and for skin rashes and gout (8). C. nutans, a polyphenolic compound derived from grapes, berries, and other plants, is acknowledged for its antioxidant features (9). The metabolites generated are distributed to various organs, involving the kidneys, liver, adrenal glands, plasma, bone marrow, and other tissues. Within these sites, they can alter lipid and amino acid metabolism, promote cytokine production, and induce systemic oxidative stress, in addition to causing renal functional disturbances, the mechanisms of which remain incompletely elucidated (10).

In the present investigation, *C. nutans* were administered to rats previously exposed to TAA to evaluate its effects on hepatic function, oxidative stress parameters, and cytokine expression. This experimental design facilitated the evaluation of the potential protective role of C. nutans in modulating TAA-induced hepatic inflammation and injury. Furthermore, the investigation aimed to explore its capacity to attenuate the detrimental consequences of oxidative stress on hepatic tissues. The outcomes from this investigation lead to a broader understanding of the molecular mechanisms involved and may support the development of targeted therapeutic strategies for liver injury.

Methods

Plant-based materials

Before being deposited in our laboratory, C. nutans was classified as a complete plant by the official scientist of the Herbal Medicine Herbarium at IBS, Karbala, after being collected from the commercial market in Iraq.

Animals

Thirty adult male albino rats (Rattus norvegicus) weighing 220 and 280 grams were procured from the Animal House of the Faculty of Science, University of Karbala. The animals were maintained under standardized laboratory conditions, including proper ventilation, a controlled 12 hrs light/dark cycle, and a 25 ± 2 °C stable temp. Throughout the experimental period, all rats had a standard rodent diet and free access to drinking water. The investigation protocols and procedures for animal care and handling adhered strictly to the ethical guidelines established by the Animal Care and Use Committee at the University of Karbala, Iraq.

The animals were randomly allocated into five experimental sets, each comprising six rats. These sets consisted of a sham control set, a vehicle control set (receiving distilled water), a TAA-treated set (administered TAA), a C. nutans-treated set (receiving *C. nutans* extract), and a combined treatment set receiving both TAA and C. nutans concurrently. The dosage of TAA administered was 10 mg/kg body weight, prepared in distilled water, and delivered via oral gavage. This dosing regimen was selected based on established protocols in previous studies (11).

Liver enzymes testing

Following blood collection from the tail vein, the serum was extracted and stored for subsequent use in a freezer set at -80°C. In regular saline, the liver was dissected, cleaned, weighed, homogenized, and sonicated with an ultrasonic cell disruptor. After that, all homogenates were centrifuged for five minutes at 4 degrees Celsius and 4,000 rpm. The resultant suspension was sonicated 4 times and then cooled to -70 degrees Celsius after centrifugation at 5,000 rpm for 360 at 4 degrees Celsius. Every single specimen was diluted to 0.01 mol/L in PBS before the experiment.

Reactive Oxygen Species (ROS) assay

The ROS test was used to evaluate the levels of superoxide dismutase (SOD), reduced glutathione (GSH), malndialdehyde (MDA), and cytochrome oxidase (COX). For the experiment, 200 µL of each specimen was introduced into a 50 mL centrifuge tube containing 800 µL of phosphate-buffered saline (PBS), 25 µL of butylated hydroxytoluene (BHT), and 500 µL of trichloroacetic acid (TCA). The solution was kept on ice for two hours to facilitate protein precipitation. Subsequent to incubation, the tubes were centrifuged at ambient temp for 15 minutes. Subsequently, 1 mL of the clear supernatant was transferred to a separate container and mixed with 250 µL of thiobarbituric acid (TBA) and 75 µL of 0.1 M ethylenediaminetetraacetic acid (EDTA) dissolved in 1 M sodium hydroxide (NaOH). The reaction mixture was heated to 100°C for 15 minutes to promote color development. After heating, the specimens were left to return to ambient temperature. Absorbance was measured at 532 nm and 600 nm wavelengths with a Beckman Coulter spectrophotometer (USA).

Assay for Cytokines

The present investigation investigates the levels of the following serum proteins and cytokines in rats: MCP-1, TGF-β, 3 Caspase, BCL2, IL-1β, IL-6, CEA, and TNF α. The specimens were collected and examined using the

DuoSet ELISA Development System (R&D Systems, USA). The production protocol executed the procedures, and the specified capture antibodies were reduced to the working amount in PBS devoid of carrier protein. In conclusion, 100 µl of diluted capture antibodies were dispensed into each well of the 96-well plates, which were then incubated at ambient temp overnight. The plates were subjected to three washings with Wash Buffer the following day. Block Buffer incubated The plates at room temperature for one hour. Subsequently, each well was filled with serum =100 µl in Reagent Diluent and incubated for two hours. After aspiration and three washing cycles, 100 µl of detection antibodies were introduced to the solutions. Subsequently, the plates were kept warm for a further two hours. Streptavidin-HRP was thereafter incubated for 120 seconds at ambient temp for every well. After three washing cycles, the plates were introduced to the Base Solution and left to react for an extra twenty minutes. Eventually, Stop Solution was introduced to terminate the reaction, and a microplate reader (Azure Biosystems) was utilized to measure the plates at 450 and 570 nm.

Statistical analysis

All data are shown as mean magnitudes ± standard error of the mean (SEM). Statistical analyses were conducted using SPSS software, version 26. Variations between sets were assessed using a one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons. A p-magnitude below 0.05 was considered suggestive of statistical significance.

Results

C. nutans modulate antioxidant activity

In the current investigation, the antioxidant impacts of C. nutans in tumors were investigated using a comprehensive assessment of COX, SOD mg/dl, MDA mg/dl, and GSH mg/dl levels. The findings obtained revealed a substantial decrease (p less than 0.05) in GSH mg/dl, SOD mg/dl, and COX levels in the TAA set when compared with the C. nutans and combination sets. As demonstrated in Table 1, GSH mg/dl, SOD mg/dl, and COX levels increased insubstantially (p higher than 0.05) in comparison with the sham and vehicle sets. Conversely, the antioxidant features of C. nutans were evaluated by measuring the MDA levels. As demonstrated in Table 1, the MDA level in the TAA set was 0.871 ± 0.11, while in the C. nutans and combination sets, the levels were 0.118 ± 0.05 and 0.386 ± 0.04, respectively.

Table 1: This table shows how markers of oxidative stress were affected by TAA on its own and combined with *C. nutans*. The data are demonstrated as the mean±SD of experiments carried out independently. Each set had six rats in all experiments. The rats were treated with TAA at 200 mg/kg for 2 weeks, with C. nutans at 10 mg/kg bw, and with TAA and C. nutans at the same time at 200 mg/kg and 10 mg/kg bw for 2 weeks.

Antioxidant markers	Sham	Vehicle	TAA (200mg/kg)	*C. nutans*	*C. nutans* +TAA	*P magnitude*
GSH mg/dl	17.03 ±0.61	16.81±1.07	9.1 ±1.83	20.35 ±2.40	16.51 ±1.03	0.0216
MDA mg/dl	0.335± 0.06	0.348 ±0.09	10.871±0.11	0.118 ±0.05	0.386 ±0.04	0.0104
SOD mg/dl	61.16± 7.42	59.33 ±4.81	21.33 ±4.93	75.83 ±4.92	58.50 ±6.01	0.0116
COX	220.816± 3.71	218.516 ±7.33	125.683 ±5.72	241. 65± 4.32	214.98 ±8.25	0.0021

C. nutans modulates inflammation and anti-apoptotic activities

The TAA set that received TAA=200 mg/kg dissolved in water for a twenty-one-day period demonstrated a substantial increase in TNF-α (ng/ml), IL-6 (pg/ml), and IL-1β (ng/L) TGF-β (ng/ ml), and MCP-1 (pg/ ml) levels. In contrast, rats that received a concurrent administration of equivalent quantities of TAA and C. nutans dissolved in water for 2 weeks demonstrated a substantial decline compared to the TAA-treated set (P less than 0.05) (see Table 2). Nevertheless, a substantial reversal was detected in the rat set that received the concurrent administration of TAA (P less than 0.05). No substantial variations were detected when the C. nutans set compared with the sham and vehicle sets (see Table 1).

BCL2 (ng/ml) levels were dramatically reduced, but Caspase 3 (ng/ml) levels were substantially elevated after TAA treatment. Nonetheless, this effect was counteracted by concurrent treatment of C. nutans with TAA. Nevertheless, treatment with C. nutans produced a statistically insubstantial change in BCL2 (ng/ml) and Caspase 3 (ng/ml) levels in comparison to the sham and vehicle sets (P less than 0.05).

Table 2: The impacts of TAA on the levels of inflammatory and apoptotic markers, both on their own and when combined with C. nutans, were investigated. The data are demonstrated as the mean±SD of experiments that were independently carried out; each set had six rats for all experiments carried out: The rats were treated with TAA at 200 mg/kg for 2 weeks, with C. nutans for 2 weeks and with TAA and C. nutans at the same time.

Parameters	Sham	Vehicle	TAA (200mg/kg)	*Clinacanthus nutans*	*Clinacanthus nutans* +TAA	*P magnitude*
TNF α ng/ml	3.06± 0.32	3.933±0.45	13.23 ±0.93	2.83 ±0.60	7.31 ±0.42	0.012
IL-6 pg/ml	35.15 ±2.54	58.116 ±9.49	96.66 ±2.51	33.81 ±4.69	57.73 ±3.91	0.001
IL-1β ng/L	87.61 ±6.72	84.816±29.81	190.016± 18.04	79.49 ±8.61	90.266±12.64	0.002
TGF-β ng/ ml	0.48 ±0.07	0.35 ±4.30	1.523 ±0.03	0.36 ±0.02	0.57 ±0.09	0.022
MCP-1 pg/ ml	45.95 ±7.41	86.91 ±31.02	147.216 ±1.85	44.07 ±3.22	47.866 ±9.59	0.011

BCL2 (ng/ml)	0.723 ±0.09	7.783 ±0.92	0.745 ±0.02	0.629 ±0.08	0.758 ±0.06	0.001
Caspase 3 (ng/ml)	5.98 ±0.17	5.9 ±1.02	26.183 ±0.74	5.28 ±0.49	16.250 ±0.18	0.032

C. nutans ameliorated liver damage activities

The TAA set, which received 200 mg/kg of TAA dissolved in water over 21 days, demonstrated a substantial increase in liver enzymes (U/L) and bilirubin (mg/dL) levels. However, rats given the same dose of TAA and C. nutans dissolved in water for 2 weeks had lower levels of these substances than the TAA-only set (P less than 0.05) (see Table 3). Interestingly, a reversal was seen in the set that received both TAA and C. nutans simultaneously (P less than 0.05). There were insubstantial variations between the C. nutans set and the sham or vehicle sets (see Table 3).

Table 3. The present table evaluates the impact of TAA on liver function test markers, both in isolation and in combination with C. nutans. The empirical design comprised independent experiments, with six rats per set. The treatments included TAA at 200 mg/kg for 2 weeks, C. nutans for 2 weeks, and a combination of TAA and C. nutans administered simultaneously for 2 weeks. The findings are demonstrated as the mean±SD.

Parameters	**Sham**	**Vehicle**	**TAA (200mg/kg)**	***C. nutans***	***C. nutans* +TAA**	***P magnitude***
ALP (U/L)	232.1 ±7.92	444.5 ±7.40	236.2 ±9.38	231.5 ±7.33	246.8±6.93	0.02
AST (U/L)	130.1±9.59	396.3±22.10	232.4 ±8.49	228.5 ±6.92	235.8 ±7.31	0.002
ALT (U/L)	46.33 ±5.31	98.66 ±4.41	47.66 ±5.21	45.61 ±5.69	48.8 ±6.02	0.011
T-BIL (mg/ dl)	0.166 ±0.04	0.673 ±0.09	0.168 ±0.04	0.162±0.07	0.171 ±0.08	0.021

Discussion

Rats that are exposed to the well-known carcinogen TAA, an organosulfur fungicide, develop centrilobular liver necrosis. It has been demonstrated that C. nutans protects against several illnesses, including diabetes, heart disease, cancer, obesity, and specific oxidative stressors (12). This investigation demonstrated that C. nutans had protective benefits against TAA-induced damage regarding cytokine release in liver tissue, DNA damage, oxidative stress, and liver function (13).

Extracts from C. nutans have demonstrated antioxidant and anticancer impacts in vitro in a range of cancer cell lines, as demonstrated in previous research. However, these extracts' antitumor and antioxidant features have not yet been fully understood in mouse models (14, 15). Consequently, the present experiments sought to explore the C. nutans leaves extracted potential to impede tumor progression in a tumor-bearing rat model. After 21 days of treatment with C. nutans, a substantial reduction in both mean ROS and inflammatory markers and liver function tests was detected.

The current investigation assessed the protective impacts of C. nutans against apoptotic and related cytokine markers (BCL2, MCP-1, TGF-β, IL-1β, IL-6, TNF-α, and Caspase 3), as well as free radicals (GSH, SOD, and Cytochrome Oxidase) and liver function markers (ALP, AST, ALT, and T-BIL). The detected rise in T-BIL, AL, AST T, and ALP levels in the TAA-treated set indicated a decrease in liver function, which is in keeping with other studies showing that acute cell necrosis is the leading cause of liver injury in these individuals (16, 17).

Response to treatment with C. nutans was illustrated to lower MDA levels; this phenomenon has been ascribed to the latter's antioxidant properties. Hepatoma cells have also demonstrated a comparable antioxidant impact based on decreased MDA levels. These findings lead one to hypothesize that C. nutans might prevent cancer cells from dying and help them survive. Several oxidative stress-induced liver damage studies have demonstrated increased lipid peroxidation (18, 19). TAA also caused higher lipid peroxidation in our research; this impact was reduced in the set receiving both C. nutans and TAA concurrently. We underline the need for co-administration with TAA exposure in suitable cases.

Conclusion

Ultimately, C. nutans leaves have anti-inflammatory and antioxidant properties that help efficiently scavenge free radicals and stop cancer development. These findings imply that the phytochemical elements included in the methanol extract might be a feasible substitute and complementing strategy for cancer prevention and therapy. Still, a more thorough investigation is required to identify the bioactive elements of C. nutans extract and clarify the processes underlying its antitumor properties, suggesting their use in cancer treatment.

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