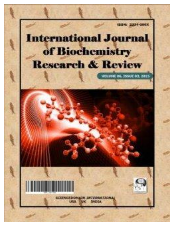

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Relevance of *Chrysanthellum americanum* (L.) Vatke Extracts in Rat Liver Protection

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Authors' contributions

This work was carried out in collaboration with all authors. Conceptualization was made by authors SG, N.Ouattara, N.Ouédraogo and AH. Data curation was performed by authors SG, PTK, NTRM, AC and MK; Formal analysis was carried out by authors SG and AH. Investigation was done by authors SG, N.Ouattara, N.Ouédraogo and AH. Methodology was performed by authors SG, N.Ouattara, AT and AH. Project was administered by authors OGN. Resources were accumulated by author PTK. Software analysis carried out by author SG. Supervised by authors AH and AT, validated by authors N.Ouédraogo, manuscript written by author SG. Written-review & edited by authors SG, N.Ouattara, NTRM, N.Ouédraogo, PTK, AC, AH and MK. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To assess the relevance of *Chrysanthellum americanum* (L.) Vatke extracts in rat liver protection.

Place and Duration of Study: Laboratory of Biochemistry and Applied Chemistry (LABIOCA), also in Laboratory of Department of Medicine and Traditional Pharmacopoeia (MEPHATRA-PH) of Institute for Research in Health Sciences (IRSS/CNRST) of Burkina Faso between July 2014 and August 2015.

Study Design: Polyphenolic extract of *Chrysanthellum americanum*- in vivo liver protection- *in vivo* liver intoxication- liver necrosis parameters analysis, histopathology analysis, *in vivo* and *in vitro* antioxidant assay.

Background: *Chrysanthellum americanum* L. (Vatke) is a medicinal plant well known for its flavonoids and saponins richness, but also for its strong antioxidant potential and use traditionally for liver disease treatment.

Methodology: *In vivo*, anti hepatotoxicity effects of *Chrysanthellum americanum* was evaluated using CCl_4 as hepatotoxic agent. Also, acute toxicities were determined using standards methods, serum parameters of liver injury using Cypress Diagnostics kits and histopathology analysis using Mayer's haematoxylin- eosin-phloxine coloration method. For in vitro tests, malondialdehyde and thiobarbituric acid method were used in lipid peroxidation assessment and the ABTS method in Trolox Equivalent Antioxidant Capacity assessing.

Results: Result showed that the crude extract of *C. americericum* has a very low oral toxicity but, in intraperitoneal route this extract presented a high toxicity (LD50= 175 mg / kg of body weight). Histopathology micrograph indicated reduction in number of necrotic cells induced by CCl_4. This beneficial action was confirmed by reduction in serum transaminases and malondialdehyde (22.68 ± 0.68 mmol MDA/ g of liver weight). In *vitro* antioxidant capacities, this plant extract presented a result of 35.01 ± 0.26 % and 42.01 ± 0.26 mg TE/ g respectively in LPO and TEAC.

Conclusion: Given our results, our research confirms that *Chrysanthellum americanum* extracts have *in vivo* physiological impact and benefits in traditional medicine for specific care of liver diseases.

Keywords: Hepatotoxicity; Medicinal plant; antioxidant; toxicity.

ABBREVIATIONS

ABTS: 2,2'-azinobis (3 ethylbenzothiazoline-6-sulphonate
ALT: Alanine Amino Transferase
AST: Aspartate Amino Transferase
C. americericum: Chrysanthellum americericum
$CCl_3^{\cdot}$: Carbon trichloride radical
CCl_4: Carbon tetrachloride
$FeCl_2$: Ferrous dichloride
GAE: Gallic acid Equivalent
HCl: Hydrochloric acid
IRSS/CNRST: Institute for Research in Health Sciences
LD: Lethal Dose
LD_{50}: Lethal Dose of 50%
LPO: Lipid Peroxidation
MDA: malonedialdehyde
MEPHATRA-PH: Department of Medicine and Traditional Pharmacopoeia
NMRI: Naval Medical and Research Institute
SD: standard deviations
TE: Trolox Equivalent
TEAC: Trolox Equivalent Antioxidant Capacity
WHO: World Health Organization

1. INTRODUCTION

Diet and good digestion are very important factors for good health and also for life good mood [1]. For this, gastroenterology diseases purpose a negative impact on the functioning of body vital organs but also on the psychology of the human being.

The liver is one of the main gastroenteric organs that has several functions of which the main ones are detoxification, synthesis (carbohydrates, lipids and proteins) and storage (vitamins A, D, E, K and glycogen) [2,3]. Being that the liver a purifying organ, its diseases are very numerous by passing from alcoholic diseases to toxic diseases and inflammatory diseases as well [4,5].

Causes of liver pathologies are several (alcohol, toxins, hepatitis virus…). However, oxidative stress is a primary factor in the appearance of these diseases with pronounced psychological effects (anxiety) [6]. Oxidative stress defined as a state of imbalance between oxidants (toxic compounds) and antioxidant defense system (molecular and enzyme) of an organism is involved in several diseases especially in metabolic diseases [7,8].

In Burkina Faso, as in most low-income countries, poverty equated with lack of hygiene keeps many people in a state of fairly high stress. In this context, the populations are subject to food and alcoholic poisoning and also viral hepatitis which have the liver as potential target.

In European countries liver diseases remain a problem [9]. Also, for WHO, hepatitis will have to be eliminated by 2030. Research to fight against liver diseases have seen many encouraging results but there are still dissatisfactions. One thing is also the high cost of treatments available for low income populations, so medicinal plants are their alternative.

Since ancient times, in African, Chinese and Ayurvedic medicines, plants have been a very important source of natural chemical compounds with enormous therapeutic potentials. Looking for remedies to establish health, researchers are turning more and more to these medicinal plants [10,11]. *Chrysanthellum americanum* (L.) Vatke is a plant used in Burkina Faso traditional medicine for its extracts antioxidant power but also well-known in herbal medicine research area.

Chrysanthellum americanum is a small erect or less prone herbaceous plant with very few leaves and yellow flowers belonging to *Asteraceae* family [12].

This plant extracts are known to possess antioxidant, P-vitamin and antilithiasis remarkable properties[13]. Most of therapeutical properties of *C. americanum* extracts are attributed to saponins (Chrysantheline A & B) and to flavonoids (luteolin 7-O-glucoside, eriodictyol 7-O-glucoside, isookanin 7-O-glucoside or flavonomarein, okanin 4'-O-glucoside or marein, maritimetin 6-O-glucoside or maritimein) of which they are consisted [13,14]. Polyphenolic compounds are well known for their antioxidant capabilities, their capacity to improve hepatoprotection [15,16,17,18].

C. americanum is a medicinal plant that its extracts are endowed with very good antioxidant capacity, but also a good candidate for treatment of pathologies related to oxidative stress [19].

Liver pathologies are disorder or diseases exacerbated by oxidative stress and affect the psychology of the patient. Thus, this present study aims to evaluate impact of polyphenolic extract of *Chrysanthellum americanum* (L.) Vatke on carbon tetrachloride hepatotoxicity on rat model.

2. MATERIALS AND METHODS

2.1 Plant Material and Extraction

Chrysanthellum americanum (L.) Vatke whole species was collected during August 2014 in Loumlila, 15 Km north of Ouagadougou, the capital of Burkina Faso. The plant was identified by Prof. Millogo- Rasolodimby from plants Biology Department of the University of Ouagadougou. A voucher specimen (ID-10474) was deposited at the Herbarium of the University of Ouagadougou.

Chrysanthellum americanum (L.) Vatke whole-plant was dried at room temperature and ground to fine powder. Seventy-five gram of this powder was macerated during 48 hours with mechanical stirring using 750 mL of aqueous ethanol (80% v/v) at laboratory conditions. After, extract solutions were concentrated under reduced pressure in a rotary evaporator (BÜCHI, Rotavopor R-200, Switzeland) at approximately 40°C, frozen and lyophilized using a lyophilizer (Telstar-Cryodos 50, Spain). The aqueous ethanol extract (Crude extract) obtained was fractionated by solvents of increasing polarity (dichloromethane, ethyl acetate, butanol and water residual). Crude extract and butanol fraction (polyphenols extract) were weighted before packed in waterproof plastic flasks and stored at 4°C until use. The yields of crude aqueous ethanol extract and polyphenols extract were 8.00% and 6.22% respectively (Yield of the crude extract was calculated with respect to vegetable powder mass and the yield of the butanol fraction relative to crude extract mass).

2.2 Animals

Thirty female and male Wistar rats weighting respectively 238.40 ± 18.70 g and 310 ± 48.60 g and mice from Naval Medical and Research Institute (NMRI) (31.83 ± 4.77 g) at the start of the experiment were used. The animals were housed in a temperature and light-controlled room (22 °C, a 12 h cycle starting at 08:00 h) and were fed with industrial pellets with 29% protein and allowed to drink water ad libitum. Rats and mice were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC). “Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee”.

All evaluations were performed between 9 h and 16 h.

2.3 Chemicals

To carry out our activities, we used analytical grade solvents and various classic reagents. Ethyl acetate and 2-thiobarbituric acid were purchased from Sigma Aldrich chemie (Steinheim, Germany); potassium persulfate, 2,2’-azinobis (3 ethylbenzothiazoline-6-sulphonate) ABTS and trichloroacetic acid were supplied by Fluka chemie (Buchs, Switzerland); dichloromethane, ferric dichloride, Carbon tetrachloride, ethanol were sourced from Probalo (Paris, France); butanol was sourced from sds (Peyin, France).

2.4 *In vivo* Experiments

2.4.1 Toxicities evaluations

The mice were randomized into groups of 6 mice (3 males and 3 females) including a control group for the crude extract of *C. americanum*. Each animal was identified by a different mark (head, back, right flank, left flank, tail and without mark). The animals were fasted for 12 hours, then the weight of each rat was taken, and they received a given dose of extract per group. The route of administrations of the extracts were oral or intraperitoneal [20]. The number of deaths per group was determined after 2h, 24h, 48h, 72h and the animals were kept under observation for a week.

50% lethal dose (LD_{50}) determination and its confidence limits is what was described by Ouedraogo [21]. It consists of directly carrying Log Probit paper the percentage of mortality according to the log of the dose. Before going to the tests, pre-tests were carried out on group of three (03) animals allowing to locate the lethal dose 50%.

2.4.2 Anti hepatotoxicity activity of *C. americanum* phenolic extract

The anti hepatotoxicity activity of *C. americanum* was evaluated according to the protocol described by Sanogo [22].

2.4.2.1 Experiment design

Rats were randomized into four (4) groups of six (6) animals:

Group I: normal control group, animals received distilled water (10 mL / kg of body weight per day) for 7 days *per os* and the 7th day received olive oil 2mL / kg of body weight intraperitoneally 1 hour after water administration;

Group II: negative control group, animals received distilled water (10 mL / kg of body weight per day) for 7 days *per os*; the 7th day received 2mL / kg of CCl_4 (50% dissolved in olive oil) intraperitoneally 1 hour after the administration of the water;

Group III: positive control group, animals were treated with silymarin (50 mg / kg of body weight) for 7 days *per os* then the 7th day received 2 mL / kg of CCl_4 (50% dissolved in olive oil) intraperitoneally 1 hour after administration of silymarin.

Group IV: test group, animals were treated with *C. americanum* phenolic extract (100 mg / kg of body weight) for 7 days *per os* then the 7th day received 2 mL/kg of CCl_4 (50% dissolved in olive oil) intraperitoneally 1 hour after administration of the extract.

2.4.2.2 Anti hepatotoxicity evaluation

On day 8th, animals were sacrificed after being anesthetized with ketamine (150 mg / kg body weight).

Biochemical analysis:

Transaminases assay: The animals' blood were collected in dry tubes, centrifuged at 3000 rpm for 5 minutes and the sera were taken to evaluate enzymatic parameters of hepatic necrosis: Aspartate Amino Transferase (AST) and Alanine Amino Transferase (ALT) using kits (Cypress Diagnostics).

Lipid peroxidation evaluation: Animals liver pieces from treated animals were removed, ground in (10% w/v) Tris-HCl buffer (50Mm, pH 7.40), centrifuged at 6000 rpm for 10 minutes, and Supernatants were used to evaluate lipid peroxidation [23].

Histopathology analysis:

Small fragments (approximately 0.2 x 0.2 cm) of liver were removed and fixed in formalin solution 10% [24]. They were dehydrated in solutions of increasing concentration of ethanol (70 to 100%) for 2 hours in each concentration. They were cleaned then in 2 xylene baths, infiltrated into 2 paraffin baths, and transferred to paraffin-filled molds. The sections of livers prepared by rotary microtome (Leitz 1512) were placed on clean slides and stained with Mayer's haematoxylin solution for 15 min, washed with water and alcohol 80% and mounted in eosin-phloxine solution. Finally, these assemblages of tissue slides were examined under an optical microscope.

2.5 *In vitro* Experiments

2.5.1 Trolox equivalent antioxidant capacity (TEAC)

ABTS radical cation decolorization assay was used to evaluate crude and phenolic extracts TEAC according to Guenné [19] with some modifications. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting aqueous ABTS stock solution (7 mM) with 2.45 mM potassium persulfate. The mixture was put down in dark at room temperature for 16 h before use. This mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm using microplates UV/visible light spectrophotometer reader (Epoch 251465, Biotek Instruments, U.S.A.). 50 µL of diluted sample (1 g/mL in methanol) were added with 200 µL of fresh $ABTS^{\cdot+}$ solution and the absorbance was taken 15 min exactly after initial mixing. Trolox was used to produce the calibration curve (R^2 = 0.99) and the antioxidant capacity of extracts were expressed as mg Trolox Equivalent per g of extract.

2.5.2 Liver lipid peroxidation inhibition

Crude and phenolic extracts lipid peroxidation (LPO) inhibitory activities were determined according to the 2-thiobarbituric acid method [25]. Ferrous dichloride ($FeCl_2$) and H_2O_2 were used to induce rat liver homogenate fats peroxidation. In this method 0.2 mL of extracts (1.5 mg mL^{-1}) was mixed with 1.0 mL of 1% liver homogenate in Tris-HCl buffer, then 50 µL of $FeCl_2$ (0.5 mM) and 50 µL of H_2O_2 (0.5 mM) were added. The mixture was incubated at 37°C for 60 min, then 1.0 mL of trichloroacetic acid (15%) and 1.0 mL of 2-thiobarbituric acid (0.67%) were added and the mixture was heated up in boiled water for 15 min. The absorbance was recorded at 532 nm using spectrophotometer. Quercetin was used as the positive controls.

2.6 Statistical Analysis

All results were expressed as mean ± standard deviations (SD). Tukey's test (one-way ANOVA) was used to determine level of significance of all results obtained on XLSTAT 7.1. Results were regarded as significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Extract Toxicities

Through oral administration, the plant crude extract showed a lethal dose of 50% (LD_{50}) greater than 3000 mg / kg of body weight because on groups of six (06) mice no mortality was observed after seventy-two hours (72 h) observation following extracts administration.

Through intraperitoneal administration, *C. americanum* hydro-ethanolic extract toxicities values were presented in the following table (Table 1).

The Log Probit paper plot of mortalities percentages based on log of dose determined *C. americanum* LD_{50} of 175 mg / kg of body weight. The line obtained has good validity because LD_{50}

/ DL_1 (2.18) is substantially equal to DL_{99} / LD_{50} (2.28) (with LD_1 = 80 mg / kg of body weight and DL_{99} = 400 mg / kg of body weight). The safety index of the extract is DL_{99} / DL_1 = 4.98 <5.

3.2 Liver Protection

3.2.1 Enzymatic parameters of liver damage

The Table 2 showed transaminases and lipids peroxidations values.

C. americanum extract has a protective effect against the oxidative aggression of carbon tetrachloride on rat livers. This effect was inferior to the beneficial effect of sylimarin, which is the reference compound used in hepatic poisoning.

Histopathology: The presence of necrotic cells due to CCl_4 (hepatotoxic agent) action and these necrosis reduction by the sylimarin or *C. americanum* polyphenolic extract actions are represented by the **photo 1**.

Carbon tetrachloride has caused hepatic necrosis (Photo 1 b) compared to normal liver (Photo 1a). Sylimarin significantly prevented the hepatic necrosis establishment (Photo 1c). This action was also borrowed by *C. americanum* (Photo 1d) extract but it remains less important than that of sylimarin.

3.3 *In vitro* Antioxidant Capacities

3.3.1 Trolox equivalent antioxidant capacity

The radical cation $ABTS^{\circ+}$ reducing power of the crude extract, polyphenol extract and quercetin are shown in Fig. 1. This figure shows that the best reducing power was obtained with quercetin (67. 99 ± 0.79 mg TE/ g) followed to the crude extract (67.53 ± 0.05 mg ET/ g) and the polyphenolic extract (42.01 ± 0.26 mg ET/ g).

3.3.2 Lipid peroxidation inhibition

Lipid peroxidation inhibition percentages of crude extract, polyphenolic extract and quercetin are shown in Fig. 1. The best inhibition percentage was obtained with quercetin and the lowest percentage with crude extract (32.60 ± 0.53%).

4. DISCUSSION

The polyphenolic extract had low antioxidant capacity in TEAC and a high capacity in lipid peroxidation compared to the crude extract of *Chrysanthellum americanum*. Our previous studies have shown that this butanol fraction was richer in total phenolic (85.65 ± 1.77 against 79.09 ± 0.80 GAE / 100 mg of extract), in flavonoids (24.03 ± 0.88 against 13.54 ± 0.44 QE) compared to the crude extract [26].

Table 1. *C. americanum* toxicity by intraperitoneal voice

			Death numbers				
Plant	**Doses**	**Mice numbers used**	**2H**	**24H**	**48H**	**72H**	**% of death at 72 H**
C. americanum	75 mg/kg	06	00	00	00	00	00
	150 mg/kg	06	00	01	01	00	33,33
	200 mg/kg	06	00	02	01	01	66,66
	250 mg/kg	06	00	03	01	01	83,33
	400 mg/kg	06	00	04	02	00	100

Table 2. Liver necrosis blood parameters

Samples	Liver weight/100 g body weight	ALAT (UI/L)	ASAT (UI/L)	lipid Peroxidation (mmol MDA/g of liver)
Control	2.67± 0.18 ^{a}	14.63± 5.71 ^{a}	20.20± 1.51 ^{a}	14.29± 0.23 ^{a}
Negative control	3.95± 0.14 ^{d}	61.96 ±13.50 ^{c}	98.16± 16.15 ^{c}	27.73± 4.13 ^{c}
Positive control	3.29± 0.21 ^{b,c}	26.80 ±14.79 ^{a,b}	49.41± 5.25 ^{a,b}	22.92±0.88 ^{b,c}
C. americanum	3.36± 0.32 ^{b,c}	58.67 ±12.62 ^{b}	60.46± 7.55 ^{b,c}	22.68±0.68 ^{b,c}

ALAT: Alanine Amino-Transferase; ASAT: Aspartate Amino-Transferase; MDA: Malondialdehyde. The results presented in the table columns with the letters a- d are significantly different at P <0.05.

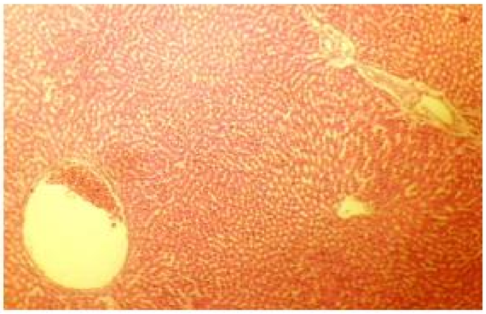

Photo 1a: Normal liver cut (X 10)

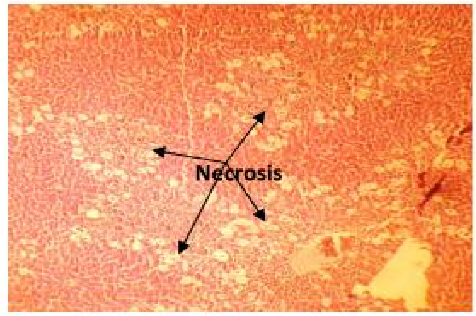

Photo 1b: CCl_4 liver treated cut (X 10)

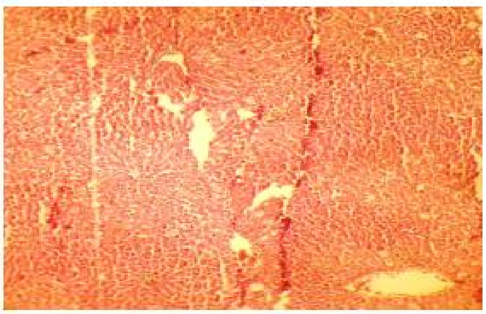

Photo 1c: Sylimarin and CCl_4 liver treated cut (X 10)

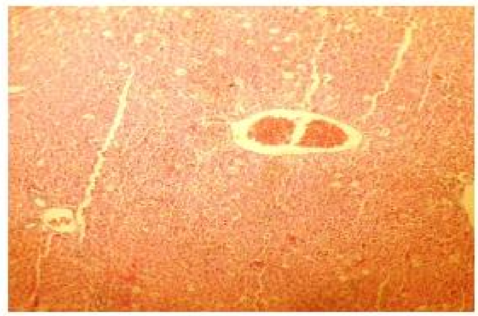

Photo 1d: *C. americanum* and CCl_4 liver treated cut (X 10)

Photo 1. Livers histopathology's analysis using photonic Microscope

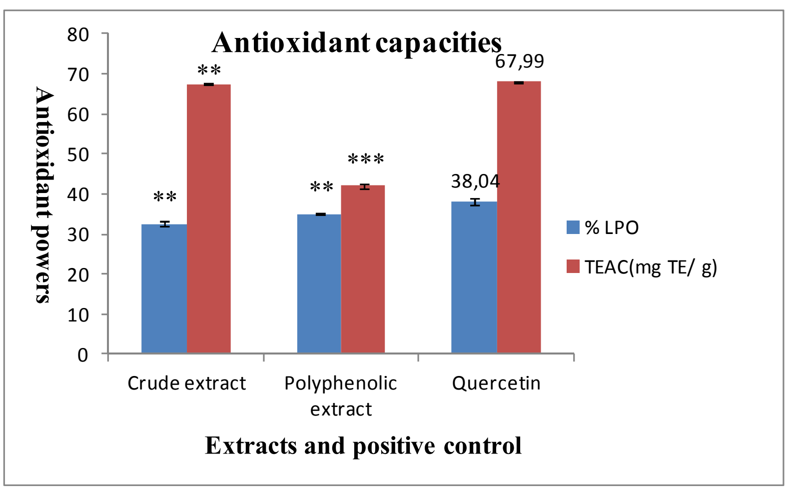

Fig. 1. Crude and polyphenolic extracts *in vitro* antioxidants powers
*The values are mean ± S.E.M. (n=3 per test). **p < 0.001 vs. quercetin and ***p<0.0001 vs. quercetin.*

Flavonoids are well known for their electron receptor hence their capacity to break the chain of free radical oxidation process [27].

It is well known and documented that a single dose of CCl_4 administration to a rat produces centrilobular necrosis and fatty degeneration of the liver. This action begins with an activation of CCl_4 and a production of $CCl_3^{\cdot}$ radical compound according to the following equation [28,29,30]:

$$CCl_4 + e^- \longrightarrow CCl_4^{-\bullet} \longrightarrow CCl_3^{\bullet} + Cl^-$$

In our study, this condition was obtained and represented with the photo 1a with necrotic cells. The richness of *C. americanum* polyphenolic extract[14] in phenolic compounds would have the advantage of protecting the liver of rats from the oxidizing action of $CCl_3^{\cdot}$. These polyphenols would inhibit CCl_4 activation or reduce the $CCl_3^{\cdot}$ radical to non-free radical compounds. Luteolin 7-O-glucoside, a flavonoid from this plant extracts was well known for its antioxidant ability. So, this molecule would has contributed to break the radical reaction or activated liver antioxidant enzymes [31,32]. Also, luteolin has a strong anti-inflammatory effects could protect rat's liver against inflammation induced by CCl_4 [33].

The result observed in photo 1d (reduction of necrosis cells number) is explained by this plant *in vitro* antioxidant activity (42.01 ± 0.26 mg ET / g and 32.60 ± 0.53% inhibition of lipid peroxidation) and also by the plant extract *in vivo* activities by reducing the blood level of transaminases and malondialdehyde (22.68 ± 0.68 mmol MDA / g of liver weight) produced by CCl_4 injection action.

Some authors have cited *Chrysanthellum americanum* extract for the treatment of kidney calculi, cholelithiasis and also as a food additive because of its richness in protein [34,36,37,17]. These properties additional to his hepatoprotection confirm this plant massive use around the world.

In addition to primary usage of this plant extract in health care with metabolic origin, Mevy group [38] found that essential oils of this plant (caryophyllene oxide, hexa-2,4-dienol, β-caryophyllene, α-pinene and verbenol) have antifungal potentials.

Our study showed that the hydroalcoholic extract of *C. americericum* has a very low oral route toxicity [39] and a high intraperitoneal route toxicity ($LD_{50\%}$= 175 mg / kg of body weight). This intraperitoneal toxicity can be explained by saponins (Chrysantheline A & B) presence in the plant extracts [40,41,42]. Fortunately, this plant is used traditionally by decoction and drink. Nevertheless, precautions are to be taken for people who would present lesions in their digestive tract.

The polyphenolic extract of *C. americanum* has a protective effect against intoxication through its antioxidant potential and has a beneficial effect on health. Also, the traditional use of this plant extract orally has virtually no toxicity.

5. CONCLUSION

Our literature review on *Chrysanthellum americanum* showed that this species has flavonoids and saponins high content and strong antioxidant capacity.

This research has made a screening of *Chrysanthellum americanum* polyphenol extract effect on rats anti hepatotoxicity using CCl_4 as hepatotoxicity agent.

The polyphenolic extract of *C. americanum* significantly prevented the oxidative aggression of carbon tetrachloride on rat liver. This beneficial action was manifested by the considerable necrotic cells number reduction and the decrease of transaminases and malondialdehyde serum levels. Our preview surveys near traditional phytotherapists of Burkina Faso central region had shown that this plant is used traditionally by decoction and per orally. This present study found also that this plant extract had a very low oral acute toxicity.

In short, our research confirms the benefits of *Chrysanthellum americanum* extracts used in traditional medicine for specific care of liver diseases.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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