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Phytochemical property and oral toxicity safety of *Chrysophyllum caimito* and *Psidium guajava* extracts

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Abstract

The century-old practice of herbal medication in animals still persists in local communities despite the contemporary advances in veterinary health care today. The numerous benefits including convenience of use, accessibility, inexpensiveness and insignificant side effects when compared to synthetic veterinary medicines in which drug resistance and residuals are linked. This research work aimed to elucidate the presence of bioactive components and determine the margin of safety of *Chrysophyllum cainito* (Caimito) and *Psidium guajava* (Guava) ethanolic bark extracts which are among the selection of documented herbals reliably utilized for gastro-intestinal sicknesses in farm animals. Qualitative phytochemical screening exemplified the presence of tannins, saponins, terpenoids, xanthoproteins, steroids and coumarin. Avian acute oral toxicity testing denoted that 2000mg/kg dosage was adequate and safe to use in Sasso chickens as substantiated by insignificant effect to body weight, SGOT and uric acid values and the non-appearance of toxicity symptoms including death. The secondary metabolites demonstrated in these plants explicate the pharmacological activities which can be utilized as potential alternatives to current medication strategies in animals.

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Introduction

Studies on phytotherapeutics are growing these days due to intimidating threats challenging animal health thus driving farmers to practice conservative animal health management which remained sustainable through the years. The low cost and no unwanted effects of the traditional preparations with the usage of curative florae make them adaptable to local community (Parthiban *et al.*, 2016). Ethno-veterinary practice irrefutably plays a part in livestock health care as an integral part of current veterinary practices (Yigezu *et al.,* 2014). Farmers used a diversity of plants and non-plant practices to treat livestock and poultry ailments. More than half of these plants were readily obtainable in their environment while 25% were rare, and thus their use is compromised (Nabukenya *et al.,* 2014).

Long before the introduction of modern medicine and westernized approaches of medications, the locals of the Philippines, have extensively been using herbs in the treatment of animal disease conditions (FAO, 1992). The Philippines is rich in biodiversity with 16, 223 identified and labelled plant species, and about 6,286 that are considered to be endemic (Catibog-Sinha & Heaney, 2006). FAO (1992) documented *Psidium guajava* and *Chrysophyllum cainito* as widely accepted herbal through all regions of the Philippines for alleviating diarrhoea in animals utilizing mostly the bark and leaf portions. Likewise, Guzman (2015) reported similar florae included in the collection of herbal plants used by Yogad people of Echague, Isabela in their ethno-veterinary treatment. Suroowan *et al.* (2017) concluded that in the case of animal health, medicinal plants can definitely be used as supportive therapy, or as preventive measures irrespective of the mode of action. This alternative medication will unequivocally have a role to play in the niche of organic farming and ultimately in modern intensive farming (Behnke *et al.,* 2008). Factors, such as the occurrence of drug resistance to pharmaceuticals are forcing to pursue alternative approaches to disease control in animals (Al-Snafi, 2016). According to FDA (2012), the mishandling and indiscriminate use of antibiotics in farm operations could result to antimicrobial resistance and the resulting failure of therapies, consequently mounting a public health problem that is of global significance. Waller (2006) highlighted that anthelmintic resistance in a problem usually associated with conventional production systems, which are more reliant on the use of anti-parasitic drugs, but less in organic systems (Giri *et al.,* 2015). Residues of the compound may occur in animal tissues if the adequate withdrawal times are not observed or if compounds are improperly administered (Posyniak & Mitrowska, 2008). The resistance has increased the need to evaluate natural products that can replace or assist current strategies of medication (Macedo *et al.,* 2012). Hence, new treatments to control infections are essential at this hour (Giri *et al.,* 2015).

Yigezu *et al.* (2014) emphasized that the traditional acquaintance in treatment of livestock diseases needs further scientific evaluations by phytochemical and pharmacological investigation to determine safety of effectiveness of use. Tropical plants with medicinal value possess dynamic bioactive components of therapeutic value (Ngbolua *et al.*, 2018*)* should be thoroughly screened of their phytochemical property (Wadood *et al.,* 2013) to qualify the ultimate beneficial usefulness as alternative remedies as well as the toxicity they cause (Confessor *et al.,* 2009). Thus this work determined the secondary metabolites present in *Psidium guajava* and *Chrysophyllum cainito* ethanolic bark extracts and find the link vis-à-vis with the ethno-veterinary uses and establish safety of dosage to animals specifically to chickens.

Materials and methods

Plant preparation

Psidium guajava and Chrysophyllum cainito were considered as plant species for assays based on the merit of being the plants of choice in the traditional treatment of animals. Sections of the selected plants were brought for authentication to the Bureau of Plant Industry (BPI), National Plant Quarantine Services Division, Regional Office 02, Tuguegarao City, Philippines.

Barks were collected from vigorous plants and then washed with distilled water (Wilkins®) to evade unwanted materials. The barks were air dried at normal environmental temperature for five (5) days. The dried barks were chopped separately into small pieces and were powdered using an electric blender. Finally, fine powder was collected through the process of sieving using a flour strainer and then sealed and labelled in separate jars stored in the refrigerator at a temperature of 4°C until use (Idris *et al.,* 2017). Ethanol plant extract was prepared by dissolving 200 grams of each powdered plant material in an Erlenmeyer flask to which 1000 ml of ethanol was added to each container. The powdered plant materials were macerated for 16 hours at room temperature of 27° C (Khan *et al.*, 2016). The opening of the Erlenmeyer flask was covered with aluminium foil and agitated for thorough elucidation of the active constituents. Extract was filtered thrice in Whatman grade number 1 filter paper. The collected extract was fed to a concentrator machine (*Genevac EZ-2 Series*) to take away the solvent. Finally, a concentrated pure crude extracts were produced and kept frozen until use.

Phytochemical screening

Samples of crude extracts were examined at the Natural Product Research and Innovation Center (NPRIC) of Cagayan State University, Philippines, for the qualitative phytochemical analyses. The evaluation was executed as follows: Alkaloids detection was carried out using 0.5 ml of plant extract added with HCl and then filtered. Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). A white or creamy white color precipitate was observed. For flavonoids, 0.5 ml of plant extract was added with aqueous NaOH and HCl. Formation of yellow to colorless solution was not found denoting non-existence of the phytochemical element. Demonstration of saponins was done using 1ml of the plant extract mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. Appearance of froth affirmed presence of the said constituent. Tannins test involved the use of 0.5ml of the plant extract added with few drops of $FeCl_3$ solution. The appearance of brownish green-black or a blue-black coloration was noted. For xanthoproteins, 1 mL of extract and few drops of nitric acid were added by the sides of the test tube. Yellow color formation was noted. Terpenoids was elucidated utilizing 1ml of extract with chloroform and a concentrated H_2S_{O4} was carefully added to form a layer. A reddish brown coloration at the interface indicates the presence of the component. As for the occurrence of coumarins, 1 ml of the extract was added with 10% sodium hydroxide in a test tube and then placed for few minutes in boiling water. The appearance of yellow fluorescence was examined. Carotenoids test was accomplished with 1ml of the sample added with chloroform, followed by vigorous shaking. The resulting mixture was filtered and 85 % of sulfuric acid was added. A blue color at the interface was noted. Presence of combined anthraquinones was examined using 1ml of plant extract added with 10% of HCl and then subjected for boiling. Cooled filtrate was partitioned with chloroform and then chloroform layer was mixed with 10% ammonia solution. Appearance of rose pink color was observed. For steroid composition, 1ml of extract was treated with glacial acetic acid and sulfuric acid. Appearance of greenish blue color was distinguished indicative of the phytochemical constituent.

Avian oral toxicity test

The procedure was assayed for the purpose of determining the safety of plant extracts to chickens following the OECD 223 guidelines specifically designed for avian species. The conduct of experimental procedures was in accordance to the protocol set by the Bureau of Animal Industy, Manila, through the endorsement of the Institutional Animal Care and Use Committee (IACUC) of Isabela State University, Philippines. The initial stock solution was prepared by weighing 1 gram (1000mg) of the crude extract to which 10 ml of acetone was added to produce 100mg/ml concentration (Zuharah *et al.,* 2015; Nagappan, 2012). Seven-week old Sasso birds pre-conditioned for two weeks in mature plumage from the same breeding population were used for toxicological assessment and distributed at random in

mixed gender into three (3) treatment groups replicated 3 times composed of 5 animals each. The groups were assigned as follows: T1- Negative control, T2- *Chrysophyllum cainito* and T3- *Psidium guajava*. As per basis of the study conducted by Abdel Aziz *et al.* (2018) employing the limit dose of 2000mg/kg in the *in vivo* anthelmintic evaluation of their subject plant, identical dosage was computed and used for evaluation. Animals for testing were individually marked with coloured nylon cable tie in their right feet at the level of the hock which served as identification mark. Prior dosing, birds were fasted for 15 hours overnight and the test extract was delivered in a single dose by gavage. Administered volume for each plant extract was given once and did not exceed 10 ml/kg BW (OECD, 2016). The negative control was given distilled water at 5ml per bird employing the same route.

Formula:

$$\text{Drug Dose} = \frac{\text{Recommended Dosage (mg/kg) X Body Weight (Kg)}}{\text{Test Drug Concentration (mg/ml)}}$$

Birds were observed continuously during the first two hours after dosing-up for possible display of regurgitation, abnormal behaviour and death and then daily observation thereafter for a total of 14 days (OECD, 2016). As part of the toxicity surveillance, weight response was monitored. Body weight of each animal was recorded prior dosing, subsequently at day 3, 7 and 14 post-administration to determine the change in body weight. In order to determine the condition of the liver and kidney being the vital organs involved for metabolism and excretion of substances, blood samples were taken from randomly selected animals for each treatment and then submitted for biochemical analysis to Best Diagnostic Laboratory located at Carig Sur, Tuguegarao City, Philippines.

In the statistical analysis, SGOT and uric acid values were analysed using One-way Analysis of Variance (ANOVA). Tukey-Kramer test was employed to assess the comparisons of means between treatments. The result was expressed as mean ± standard error of mean. All results were considered statistically significant at 5% level. Statistical program used was NCSS Version 12.

Result and discussion

Phytochemical screening

Qualitative phytochemical screening (Table 1) revealed that ethanolic bark extract of *Psidium guajava* has high concentration of tannins (Mishra *et al.*, 2017; Ekeleme *et al.*, 2017; Kamath *et al.*, 2008; Ngbolua *et al.*, 2018), saponins (Mishra *et al.*, 2017; Kamath *et al.*, 2008; Ngbolua *et al.*, 2018) and terpenoids (Mishra *et al.,* 2017; Gayathri and Kiruba, 2014; Ekeleme *et al.*, 2017; Kamath *et al.*, 2008), moderate concentration of xanthoproteins and scarce levels of steroids (Mishra *et al.*, 2017) and coumarin. Whereas *Chrysophyllum cainito* ethanolic bark extract is abundant with saponins (Doan *et al.*, 2018) and terpenoids (Shailajan *et al.*, 2014), moderate levels of tannins (Shailajan *et al.* 2014; Doan *et al.*, 2018) including xanthoproteins and low level of coumarin.

Table 1. Summary of demonstrated secondary metabolites.

Bioactive Substance Tested	Test Result	
	Psidium guajava	*Chrysophyllum cainito*
Tannins	+++	++
Saponins	+++	+++
Flavonoids	-	-
Anthraquinones	-	-
Alkaloids	-	-
Terpenoids	+++	+++
Carotenoids	-	-
Steroids	+	-
Coumarins	+	+
Xanthoproteins	++	++

- (absent); + (low concentration); ++ (moderate concentration); +++ (high concentration)

Some of the demonstrated secondary metabolites have known biological functions. Tannins are documented to inhibit enzymes, break plasma membrane and dissociate microbial substrate (Pereira *et al.*, 2015), an specific activity against bacterial agents (Bhagavathy *et al.*, 2018), adversely distressing the integrity of the cuticle of larvae and adult nematodes (Fernandez *et al.,* 2014 ;) and ova hatching inhibition capacity (Molan *et al.* 2000). Equally, saponins have special effects on the cell membranes which result in deterioration and subsequent surge of cell permeability of parasites which explicates the nematotoxic effects (Botura *et al.,* 2013; Argentieri *et al.*, 2008), stopping of egg hatching and

casting of larvae (Doligalska *et al.*, 2011), also as anti-tumor, anti-insect (Dixon and Sumner, 2003), hepatoprotective and haemolytic activities (Bink *et al.*, 2011). Whereas terpenoids are observed to be active against a range of organisms because of the synergy of several terpenoids which can act on several targets as they are a complex mixture of compounds that can interact with numerous molecular targets (Katiki *et al.*, 2013) and activity against Gram (+ and -) bacterial strains concentrations (Weli *et al.,* 2018).

Avian acute oral toxicity test

Study findings indicated that neither of the orally dose Sasso chickens with the prepared plant extracts has suffered to death within the prescribed 14-day observation period, nor manifested any adverse reactions such as regurgitation including abnormal demeanour that would denote intoxication. Weight response signified that there were no significant differences on the weight of birds from day 3, 7 and 14 among the groups (Table 2). Result specifies that *Psidium guajava* and *Chrysophyllum cainito* ethanolic bark extracts have no adversarial effects to Sasso chickens at 2000mg/kg dosage.

Table 2. Mean bodyweight (kg) transformation of birds for three consecutive surveillances.

	Weight Difference (kg)			
Treatments	*Day 0*	*Day 3*	*Day 7*	*Day 14*
T1 (Negative Control)	0.96 kg	1.11 (± 0.14) [a]	1.285 (± 0.20)[a]	1.54 (± 0.23)[a]
T2 (*Chrysophyllum cainito)*	0.92 kg	1.05 (± 0.12) [a]	1.285 (± 0.21)[a]	1.63 (± 0.20)[a]
T3 (*Psidium guajava)*	0.99 kg	1.15 (± 0.19) [a]	1.34 (± 0.25)[a]	1.57 (± 0.25) [a]

Common superscript letters indicate no significant differences

In order to validate possible concealed internal alterations to vital organs, selected kidney and liver function tests were performed (Table 3). Result inferred that the level of aspartate aminotransferase (AST/SGOT) has no significant statistical variation with the negative control (T1), although slight escalation was noted for the generated values in *Chrysophyllum cainito* (T2) and *Psidium guajava* (T3) with reference to the normal value of 70-220*u*/L which biologically signifies that the liver was physiologically challenged during the 14-day monitoring period. Equally, the level of uric acid in all treatment groups did not show significant differences as substantiated by the normal reference value of 113-743 *umol*/L. The generated result indicates that Sassso chickens can tolerate 2000mg/kg dosage of the plant extracts without deleterious effects on the liver and kidney functions as corroborated by nonappearance of symptoms or signs specific to these organs. Therefore, it can be judged at the 95% confidence level that the median lethal oral dose (LD50) of both plants for Sasso chickens is above the aforementioned limit dose of 2000mg/kg-bwt (OECD, 2016). The findings generated herein coincide with the toxicity studies conducted in aqueous guava leaf extract using mice and rats at 5000mg/kg dosage (Ngbolua *et al.*, 2018) and guava leaf oil in brine shrimps at 4mg/*ul* dilution (Weli *et al.*, 2018) recording no toxic effects to these animals. Similarly, related study on ethanol and aqueous cainito leaf extracts did not cause any mortality to albino wistar rats at different concentrations (Shailajan *et al.*, 2014).

Table 3. Mean SGOT and uric acid values of Sasso chickens.

	Biochemical Parameter	
Treatments	SGOT/AST (*u*/L SI)	Uric acid (*umol*/L SI)
Reference Values	70 - 220 *u*/L*	113 - 743 *umol*/L **
T1 (Negative Control)	214.83 (± 7.44)[a]	161.09 (±33.11)[a]
T2 (*Chrysophyllum cainito)*	224.05 (± 4.10)[a]	211.67 (±18.19)[a]
T3 (*Psidium guajava*)	226.78 (±13.64)[a]	194.27 (±47.31)[a]

Common superscript letters indicate no significant differences

*Reference values of Melluzi et al (1991)

**Reference values of Jain (1993)

Conclusion

The use of *Chrysophyllum cainito* and *Psidium guajava* in ethno-veterinary treatment of gastro-intestinal disorders in animals may hold a logical basis, as this study had elucidated the presence of a

number of secondary metabolites with known pharmacological activity such as being anthelmintic, antibacterial, anti-oxidant, anti-inflammatory and many more, which would explain the curative effect to sick animals as claimed by ethno-veterinary practitioners. The utilized limit dose at 2000mg/kg was adequate and safe to chickens. Hence the documented plants may share for the advancement of phytotherapeutic natural products recognizing the dilemma on drug residues and resistance that is currently challenging the animal industry.

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Abbreviations
Aspartate A minotransferase (AST)
Serum Glutamic-Oxaloacetic Transaminase SGOT
Organisation for Economic Co-operation and Development (OECD)

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