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Willow (*Salix acmophylla* Boiss.) Leaf and Branch Extracts Inhibit In Vitro Sporulation of Coccidia (*Eimeria* spp.) from Goats

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Abstract: Willow (*Salix* spp.) trees, found worldwide, contain secondary metabolites that are valuable as dietary supplements for animal feed and as antiparasitic compounds. We quantified secondary metabolites (phenolics, flavonoids, and salicylic acid) in ethanolic extracts from leaves and branches of three *Salix acmophylla* Boiss. genotypes and investigated their potential to inhibit *Eimeria* sp. sporulation, a major concern in ruminants. The total phenolic content of willow leaves and branches was similar in two of three different genotypes. The total flavonoid content of the branches was significantly higher than that of leaves of the same genotype; however, the salicylic acid content was significantly higher in leaves than in branches. Importantly, all extracts exhibited significant inhibition of *Eimeria* sporulation, where over 70% inhibition was obtained at concentrations as low as 750 mgL^{-1}. The sporulation inhibition by branch or leaf extracts exceeded 80% for leaves and 90% for branches at concentrations above 1250 mgL^{-1}. The study highlights the potential of using *Salix* extracts as bioactive compounds for biological control of coccidiosis in ruminants. We conclude that all parts and all investigated genotypes of *S. acmophylla* can provide secondary metabolites that act as a coccidiostat to treat *Eimeria* in goats.

Keywords: ethanol extracts; plant secondary metabolites; coccidia sporulation; *Eimeria* sp.; willow genotypes

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1. Introduction

Willows (genus *Salix)*, the largest woody species in the Salicaceae family, thrive in both natural and cultivated habitats across numerous countries worldwide [1]. The trees encompass up to 500 species and more than 200 hybrids, characterized by significant variability in size, growth rates, and plant architecture [2,3]. Willows have been used worldwide as a traditional remedy for more than 6000 years [4].

Willow exhibits high rates of evapotranspiration and a robust tolerance to soil water saturation, which can lead to oxygen shortages in the root zone [5]. These trees are distinguished by their rapid growth rates, substantial biomass yield across multiple harvests, adaptability to dense plantings, and ability to regenerate from the stump after numerous harvests [6]. Nutritionally, willow leaves and fine branches offer richer sustenance for grazing ruminants compared to low-quality summer pastures [5,7]. Beyond nutritious forage, the trees yield secondary compounds such as anthelminthics, antioxidants, and analgesic secondary metabolites [8–10]. Secondary metabolites can protect plants against herbivores, bacteria, viruses, or competing plants, and act as signaling compounds to attract pollinators or facilitate seed dispersers. Thus, these secondary metabolites are not only crucial for the survival and reproductive fitness of the plant but can also serve as adaptive components subject to natural selection throughout evolution [11,12].

Secondary metabolites in willows are primarily composed of phenols and phenolic glycoside compounds. Phenolic compounds, based on their chemical structure, can be categorized into subgroups such as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids [13]. Phenolic glycosides, especially salicylates, have been utilized by humans as remedies for pain relief, fever reduction, and the alleviation of rheumatic disorders, inflammations, and headaches [8]. Salicin, the most prevalent phenolic glucoside in willows, is converted into acetylsalicylic acid, the nonsteroidal anti-inflammatory drug commonly known as aspirin [14,15]. Salidroside, also found in willow, attenuates inflammatory responses by suppressing nuclear factor-κB and mitogen-activated protein kinase activation in lipopolysaccharide-induced mastitis in mice [16]. Animal health can also be improved by treatment with salicylates. For example, administering sodium salicylate to cows in early lactation had beneficial effects throughtout the whole lactation period [17].

At least 36 different chemical compounds were identified in willow, mainly using ethanolic plant extracts, some of which exhibit antioxidant, antimicrobial, and cytotoxic activities against human leukemia cells (HL 60) [18–21]. While the growth of willow plants may be influenced by water sources, their chemical content remains unaffected [6]. Furthermore, secondary metabolites in willow plants offer a promising alternative to conventional drugs for treating parasitic nematodes in livestock [22–26]. Most research has focused on *S. alba* (native to Europe and Asia) and *S. babylonica* (native to China), with relatively little investigation into *S. acmophylla*, which is native to the Middle East. Four *Salix* species of willow were reported in Israel by Rottenberg, et al. [27]. However, a more recent survey by Muklada et al. [28] analyzed willow samples from both Jordan and Israel, identifying only two species, pure *S. alba* and pure *S. acmophylla*, and hybrids in which the genetic majority is *S. acmophylla* with minor genetic contributions from *S. alba* and other unidentified *Salix* species. The current study utilized the *S. acmophylla* Boiss. genotypes as defined in [28].

Salix acmophylla biomass is composed of 45% leaves and 55% branches, on a dry matter (DM) basis [6]. The nutrient and mineral contents, as well as the concentration of secondary compounds in branches and leaves, varied between two willow phenotypes, initally termed “red” and “white” for their bark color. “White” willows contained 1.2-fold more salicin, 1.5-fold more gallic acid, and 1.8-fold more kaempferol than “red” willows. The two types of willow did not differ in concentrations of salicylic acid, hyperin, salidroside, and helicon. Short-term feeding of dairy goats with willow fodder might promote anti-inflammatory effects and/or delay of mammary involution [6]. Subsequent research revealed that *S. acmophylla* has three genotypes [29], which have yet to be investigated regarding their metabolic and coccidistatic characteristics.

In ruminants, coccidiosis is a parasitic disease caused by *Eimeria* spp., which has a significant economic impact due to its global distribution and high infection rates, sometimes exceeding 90% incidence in certain areas [30–33]. The primary clinical symptom of coccidiosis is diarrhea, but under conditions that are favorable for *Eimeria* development, other accompanying clinical symptoms can include low feed conversion rate, weight loss, and even death, leading to substantial economic losses from increased veterinary costs and high mortality rates among infected animals [32,33]. Kids and lambs between one and four months of age are the most vulnerable compared to older animals [30,31,33]. While coccidiosis can affect all livestock species, it is important to note that coccidia is host-specific, meaning that the coccidia found in cattle or chickens are specific to those animal species and do not cause disease in goats and sheep, or vice versa. Rivero-Perez et al. [34] identified a significant inhibitory effect (97% in vivo) of an ethanolic *S. babylonica* extract on *Eimeria* sp. coccidia in rabbits at doses ranging from 25 to 50 mg/kg body weight.

In pursuit of a natural source of bioactive compounds for the biological control of coccidiosis in goats, we extracted secondary metabolites from leaves and branches of three different *S. acmophylla* genotypes and evaluated their potential to inhibit the sporulation of *Eimeria* oocysts found in goats.

2. Materials and Methods

2.1. Plant Material and Sample Preparation

From a collection of more than 40 ecotypes sampled in different regions in Israel and Jordan in 2013–2017, three genotypes of *S. acmophylla* were identified based on principal component analysis, which was performed through DNA extraction and sequenced using a genotyping-by-sequencing process [28]. Willows collected from native stands across various regions of Jordan were planted in replicated blocks in a 0.5 ha plot at the Deir Alla Agricultural Resarch Center in the Jordan Valley in 2021, as part of a joint Jordanian–Israeli research project funded by USAID. Genotype 1 is pure *S. acmophylla*, genotype 2 is a 60:40 mix of *S. acmophylla* and another unidentified *Salix* species, and genotype 3 is a 50:50 mix of *S. acmophylla* and *S. alba* [28]. In June 2023, foliage was collected from Deir Alla and separated into leaves and thin branches up to 12 mm in thickness. This included genotype 1 (a mixture of ecotypes from Ain Fotaha, Rajab, and Shoubak), genotype 2 (a mixture of ecotypes from Ajloun and Wadi Kursi), and genotype 3 (a mixture of ecotypes from Rmimmen and Hajleh). The samples were initially dried under shade for a few days and subsequently dried for 48 h in an oven at 60 °C. The dried materials were ground into composite samples for each genotype, stored in paper bags at room temperature (23–26 °C), shipped to Israel, and maintained at 20–22 °C until use.

2.2. Quantity and Quality of Secondary Metabolites in Willow Leaves and Branches

2.2.1. Preparation of Plant Extracts

Samples were prepared as previously described [26,35]. Five grams of ground dried leaves or branches were incubated at room temperature, 23–25 °C, with 50 mL of 7:3 ethanol/water (*v*/*v*) for 24 h. The mixture was then filtered, and the solvent evaporated under a vacuum (Rotorvapor Hie-VAP; Hiedolph, Schwabach, Germany) at 40 °C to remove the ethanol and water. The yield of the extract was calculated by dividing the weight of the leaf or branch extract (g) by the weight of the leaves or branches before extraction (g) and expressed as g of extract per g of dry matter of plant biomass, in percentages. The extract was stored at −20 °C until it was analyzed or used.

2.2.2. Phenolic Acids and Flavonoid Assays

The total phenolic acid content in plant extracts was evaluated and calculated as equivalents of gallic acid (GAE) or tannic acid (TAE) according to the Folin–Ciocalteu method as described by Zhishen et al. [36]. Salicylic acid content was determined according to Warrier et al. [37], and flavonoid content according to Zhang et al. [38].

2.2.3. Coccid Sporulation Test

The effect of plant extracts from the three different willow genotypes was tested in vitro to assess their impact on coccid sporulation. Oocysts of *Eimeria* sp. were isolated from feces collected from goats that had spontaneously become infected during unsupervised grazing. The flotation method of Ekawasti et al. [39] was adopted with some modifications for oocyst purification by using a saturated sucrose solution (specific gravity 1.27). A 3.5 g sample of goat feces was lightly crushed, suspended in 50 mL of tap water, and filtered through a 500-micron (35 mesh) sieve. The filtrate was centrifuged at 1700 rpm for 3 min at 4 °C. The supernatant was discarded, and the sediment was suspended in a 50 mL

4:3 sucrose/water solution and allowed to stand for 5 min to allow coarse fecal material to sink. The suspension was again centrifuged at 1700 rpm for 3 min at 4 °C. A quantity of 1 mL of the sample solution was placed onto a McMaster Chamber at a magnification of 40X to count *Eimeria* sp. oocysts in the entire field. When oocysts were detected, approximately 7 mL of the oocyst suspension was aspirated from the 50 mL test tube surface and then washed twice in phosphate saline buffer (pH 7.3) using centrifugation at 1700 rpm for 3 min at 4 °C. The supernatant was discarded, and the oocysts were concentrated by re-suspending the sediment in a buffered saline solution. The oocysts were stored at 4 °C to prevent sporulation from occurring until they were used for the in vitro sporulation assay.

The sporulation test was conducted in 0.5 mL centrifuge tubes containing a total volume of 100 µL of extracts at concentrations of 250, 750, 1250, and 5000 ppm, diluted from stock extracts (leaf or branch) from each willow genotype. The 100 µL of total volume included a fixed volume of 30 µL of oocyst suspension containing approximately 15×10^3 oocysts. The control group consisted of oocysts suspended in buffer alone.

The tubes were perforated to facilitate oxygen delivery and were incubated for 48 h at 28 °C with constant shaking at 150 rpm to promote sporulation. After the control group reached 90% sporulation (within 2–3 days), the effects of willow extracts on oocyst sporulation were examined using a light microscope (Motic Laboratory Microscope BA210E; San Francesco, CA, USA) at a magnification of 40X, using 10 µL of each suspension.

Sporulated oocysts were counted, and the percentage of sporulation inhibition (SI) was calculated using the following two equations:

$$\text{Sporulation}(\%) = \frac{\text{number of sporulated oocysts}}{\text{total number of oocysts}} \times 100$$

$$\text{SI}(\%) = \frac{\%\text{Sporulation of Control} - \%\text{Sporulation of Treated}}{\%\text{Sporulation of Control}} \times 100$$

where SI(%) is the percentage of inhibition of the sporulation.

2.3. Data Analysis

The experiment was performed twice in its entirety, with three replicate samples each time. Statistical analysis was carried out using PRISM 8 statistical software. A two-way ANOVA was utilized to assess the significance of the data in the study ($p \leq 0.05$). Means were separated using Tukey's HSD test.

3. Results

3.1. Yield (%) of Ethanolic Extracts from Willow Leaves and Branches

The dry extract yield of willow leaves from the three different genotypes (L1, L2, and L3) averaged 31.3%, nearly 2.5 times greater than that of the branch extracts (B1, B2, and B3) (Table 1).

Table 1. Ethanol (70%) extraction percentage and chemical components in willow leaves and branches of different *S. acmophylla* genotypes. Top of Form.

Willow (Salix) Plant Tissue/Genotype	Extraction Yield (%) ±2Std	Total Phenols		Total Flavonoids	Salicylic Acid Content
		GAE * (mg)/Extract (g) ±2Std	TAE ** (mg)/Extract (g) ±2Std	Rutin (mg)/Extract (g) ±2Std	Salicylic Acid (mg)/Extract (g) ±2Std
Leaf 1	32 ± 0.00	72.3 ± 4.12 b ***	64.9 ± 3.6 b	129 ± 2.31 a	59.8 ± 0.76 b
Leaf 2	31 ± 0.00	71.3 ± 2.72 b	64.1 ± 2.45 b	110 ± 9.25 b	59.4 ± 2.25 b
Leaf 3	32 ± 0.01	83.5 ± 0.98 a	75 ± 0.88 a	128 ± 2.31 a	75 ± 1.44 a
Branch 1	12 ± 0.00	93 ± 4.68 a	83.5 ± 4.2 a	210 ± 1.16 a	40 ± 1.83 b

Table 1. *Cont.*

Willow (Salix) Plant Tissue/Genotype	Extraction Yield (%) ±2Std	Total Phenols		Total Flavonoids	Salicylic Acid Content
		GAE * (mg)/Extract (g) ±2Std	TAE ** (mg)/Extract (g) ±2Std	Rutin (mg)/Extract (g) ±2Std	Salicylic Acid (mg)/Extract (g) ±2Std
Branch 2	13 ± 0.01	78.1 ± 9.50 b	70.1 ± 8.5 b	165 ± 16.19 b	44 ± 4.33 ab
Branch 3	11 ± 0.01	86.3 ± 2.34 b	77.5 ± 2.1 ab	177 ± 12.72 b	52.1 ± 0.38 a

* GAE—gallic acid equivalent; ** TAE—tannic acid equivalent. Means and standard deviation of two experiments, each with three replicates, mean separation in each tissue by Tukey's HSD test $p \leq 0.05$. *** Different letters indicate significant differences at $p \leq 0.05$.

3.2. Total Phenolic Content of Willow Leaf and Branch Extracts Obtained from the Different Genotypes

The total phenolic content (TPC) of both leaf and branch extracts showed little variations whether measured as GAE (gallic acid equivalents) or as TAE (tannic acid equivalents) (Table 1). Leaves from genotype 3 contained 17% more TPC than leaves from the other two genotypes. Branches of genotype 1 had approximately 17% more TPC than those from genotype 3, with the values for genotype 2 being intermediate (Table 1).

3.3. Total Flavonoid Content of Extracts from Leaves and Branches of the Different Willow Genotypes

The total flavonoid content (TFC) of leaves from genotypes 1 and 3 was similar and was 17% greater than the TFC of genotype 2 (Table 1). Branch extracts of genotype 1 had 24% more TFC than those of the other two genotypes. Branch TFC exceeded that of leaf TFC by 63%, 50%, and 38%, for genotypes 1, 2, and 3, respectively (Table 1).

3.4. Salicylic Acid Content of Willow Leaves and Branches

The salicylic acid (SA) content of leaves was higher than that of branches by 50%, 34%, and 44% for genotypes 1, 2, and 3, respectively (Table 1). The SA content of leaves from genotype 1 was 27% higher than that of the other two genotypes, while the SA content in branches of genotype 3 was 33% higher than that of genotype 1, with genotype 2 being statistically comparable to the other two.

3.5. Anticoccidial Activity of Willow Extracts Obtained from Leaves and Branches at Different Concentrations of the Three Genotypes

Leaf and branch extracts from the three different willow genotypes exhibited similar levels of sporulation inhibition percentages at a given concentration with no significant differences (Figure 1). Increasing the concentration of the extracts from 250 mgL^{-1} to 750 mgL^{-1}, 1250 mgL^{-1}, or 5000 mgL^{-1} significantly increased sporulation inhibition from approximately 35% to 75% at 750 mgL^{-1} and to more than 90% at both 1250 and 5000 mgL^{-1}.

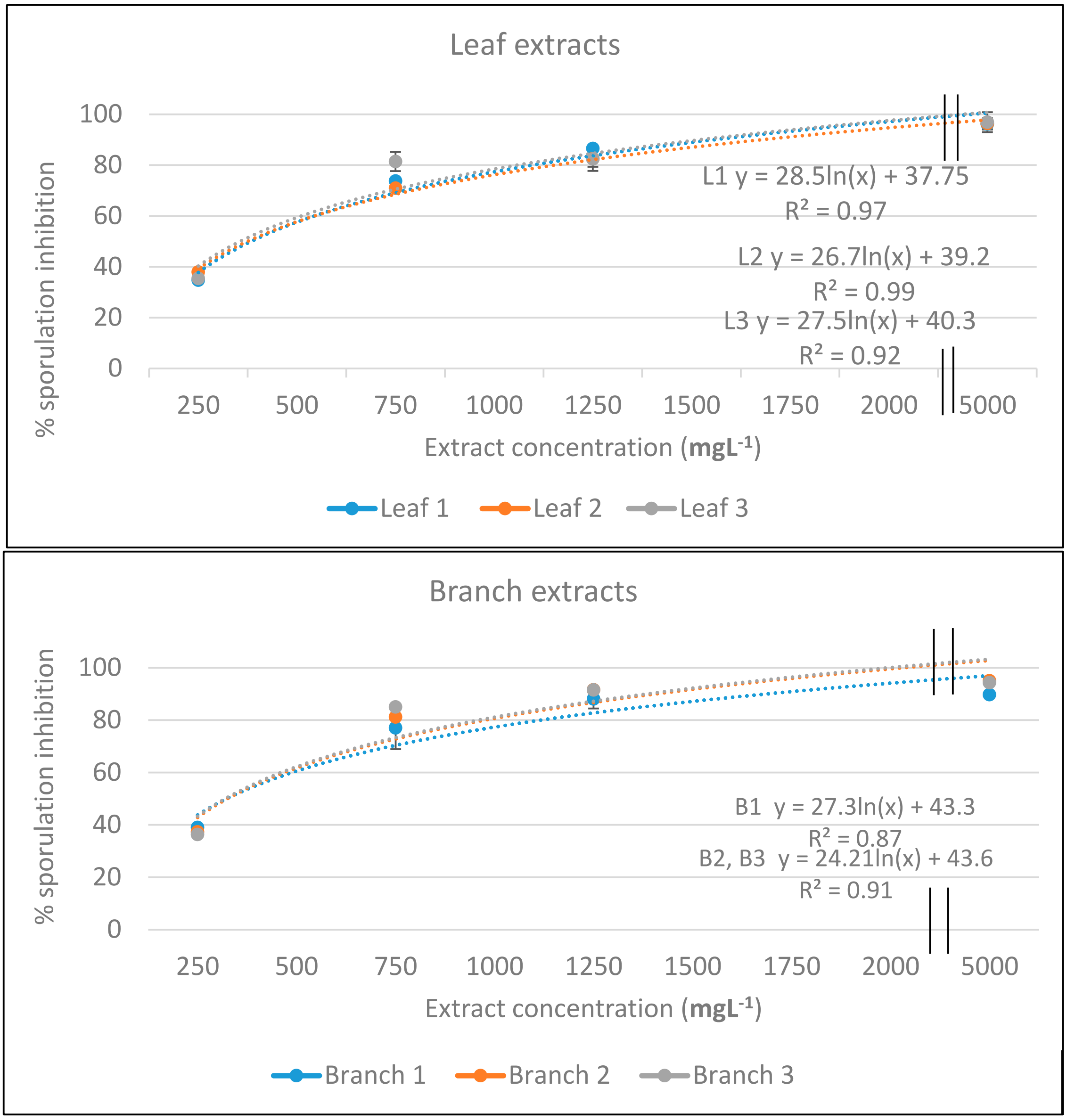

Figure 1. Percent inhibition of *Eimeria* sporulation by ethanolic extracts from leaf (**above**) or branch (**below**) tissues from the three genotypes of *S. acmophylla*. Each data point is the mean of two triplicate experiments. Standard deviation is shown where it is larger than the data point.

4. Discussion

The phytochemistry of the Salicaceae family has been under investigation since the 19th century, initially focusing on various pharmaceutical applications, particularly related to the production of salicylic acid [13,26,40]. Subsequently, ecophysiology emerged as a significant area of study. These efforts have culminated in comprehensive knowledge regarding the secondary metabolite content of Salicaceae, including flavonoids, phenolic and nonphenolic glycosides, procyanidines, organic acids and their derivatives, simple phenolics, sterols and terpenes, lignans, volatiles, and fatty acids [8,13,26,40]. These substances have attracted considerable attention for their role in plant–herbivore interactions [9]. Willow extracts, in particular, have been recognized for their anti-inflammatory, antioxidant, antirheumatic, antipyretic, antibiotic, antidiabetic, anti-parasitic nematodes, and antiseptic properties, which can vary by genotype [26,40–42].

Our results are the first publication of the extract yield, the total phenolic content, flavonoids, and salicylic acid, and the percentage of sporulation inhibition in genotypes of *S. acmophylla*. The extract yield of leaves was more than 2.9-fold greater than that of branches. Salicylic acid levels in leaves exceeded those in branches by up to 1.4-fold. However, branches contained as much as 2.6 times more total flavonoid content than that of leaves. Nonetheless, the total phenolic content (TPC) was similar in both leaves and branches across the three tested genotypes. In our previous work, the source of irrigation water (treated wastewater or freshwater) did not influence the quantity and quality of secondary metabolites, such as phenols, in willow tissue, but there were differences in the chemical profile of glycoside phenols between willow genotypes [26].

Despite variations in secondary compound content between leaves and branches, sporulation inhibition percentages were similar (Figure 1). The TPC indicates the overall phenolic content in an extract. Preliminary analysis revealed significant differences in concentrations for similar phenols or flavonoids between branches or leaves, as well as some different phenolic compounds which require further analysis. Salicylates, a group of chemically related phenolic glucosides based on the structure of saligenin (salicyl alcohol), are the most common phenolic glucosides found in the Salicaceae family, particularly in willow, aspen, and poplar [41,43]. To date, more than 20 different phenolic glycosides—glycosylated and esterified derivatives of salicyl alcohol—have been described and found in variable concentrations in members of the Salicaceae [41]. Several of these compounds have shown bioactive properties against insects [44] and against nematodes in goats [26].

Extracts of *S. babylonica* have been extensively utilized as anthelmintics and were found to be effective against gastrointestinal parasites in sheep and goats, including *Eimeria* [24,45]. The extracts were orally administered to each animal weekly before the morning feeding for 60 days, during which fecal eggs or oocysts of *Eimeria*, *Dictyocaulus*, and *Moniezia* were counted [45]. For sheep, the administration of plant extracts significantly decreased the fecal egg count [45]. After 20 days of treatment, eggs or oocysts of most parasites were not detected. It was concluded that the weekly administration of the plant extract can be effectively used to treat gastrointestinal and lung nematodes of small ruminants in both organic and traditional farming systems in tropical regions; however, the efficacy in the tested sheep was higher than in goats [45].

Coccidiosis poses a tremendous burden in modern animal farming as it lowers the productivity of animals, which in turn ultimately increases the economic burden on the farmers [46]. Botanical formulations of various plants and plant parts containing flavonoids, alkaloids, tannins, terpenes, sulfur compounds, and others were proven effective against ruminant coccidiosis-causing agents [47–49]. The antioxidant, antimicrobial, and immunomodulatory activity of these formulations can suppress or down-regulate development of *Eimeria* [48,49]. For further progress in the treatment of protozoal diseases like coccidiosis, medicinal plants that possess therapeutic potential should be sought out and investigated for their antiprotozoan activity [50]. The rate at which resistance is developed by pathogens to antimicrobial drugs is higher than the pace of development of new and effective drugs [51,52].

The development of drug resistance, drug residues in food of animal origin, consumer demand for organic animal products, the decrease in the effectiveness of synthetic drugs, and the urgent need to find alternative control methods all mandate research-based discovery efforts using plant-based preparations. Active compounds isolated from willows and other plants were found as promising alternative sources to synthetic drugs to control nematodes, coccidiosis, and other parasites in ruminants [26,47]. Extensive research, isolation, and screening are required to discover new plant compounds having antiparasitic activity and their mode of action in ruminants. We previously showed that silage made from *S. acmophylla* irrigated with saline water does not adversely affect milk quality or chemistry in goats [53], while feeding sheep willow silage made from trees irrigated with treated wastewater led to more tender meat [54]. This report is the first to explore the potential of *S. acmophylla* extracts as bioactive compounds for the biological control of coccidiosis in ruminants. Both leaves and branches of *S. acmophylla* contain secondary metabolites that act as coccidiostats for treating Eimeria in ruminants (Figure 1). In an in vivo study using willow, the anthelmintic effects of oral administration of *S. babylonica* L. (SB) and *Leucaena leucocephala* Lam. (LL) application of 30 mL crude extract per day for 63 days reduced egg and worm counts in lamb feces by 40–54% for LL versus the control lambs [24]. This in vivo study was done with crude extracts and there was no evaluation or quantification of bioactive compounds [24]. Other in vivo studies in goats using silage or biomass of *S. acmophylla* resulted in more tender meat and did not adversely affect milk quality or chemistry [53,54]. The current study is the first to quantify in vitro total phenolic content, flavonoids, and salicylic acid, and the percentage of sporulation inhibition in genotypes of *S. acmophylla*. These results are the basis for an in vivo study that will be performed using Salix extracts as coccidides in goats and other ruminants.

Preliminary results indicate variability in the secondary metabolite profiles between the extracts obtained from leaves and branches across different genotypes, although all extracts are active coccidiostats. Further research will identify and characterize the chemical properties of the specific secondary metabolites responsible for biological activity. It is also important to investigate the effect of the irrigation source on willow (saline or treated wastewater vs. fresh water) on the composition of secondary metabolites and their impact on *Eimeria*, similarly to studies conducted with nematodes [26]. Additionally, the Israeli ecotypes within each genotype warrant further examination, as well as the validation of our in vitro results in vivo. The utilization of secondary metabolites from plants offers an environmentally friendly approach to controlling parasitic coccidia in animals while maintaining or improving milk and meat quality.

5. Conclusions

At a concentration of 750 mgL^{-1}, leaf or branch extracts from three Jordanian *S. acmophylla* genotypes inhibited coccidia sporulation by more than 70% in vitro. The inhibition percentages increased to over 80% at 1250 ppm and exceeded 90% at 5000 mgL^{-1}. The phenolic composition of each extract was found to be sufficient for effective inhibition of sporulation. Although this study did not pinpoint the specific phenolic and flavonoids responsible for this inhibition, it suggests that using either branches or leaves can achieve a comparable result. Leaves and branches from willow plants of the three different genotypes can be collectively utilized to derive secondary metabolites acting as a coccidiostat to treat *Eimeria*.

Author Contributions: M.H.-Z.: Methodology, original draft, data analysis, supervision of the research. N.M.: Conducting the experiments, data curation. S.A.: Collection, preparation, and analysis of willow plant materials. R.S.: Cultivation of willow genotypes, data analysis. A.M.: Collection of coccids from goats, performance and data analysis of sporulation experiments. J.D.K.: Funding acquisition, review and editing of the manuscript. H.A.: Conceptualization and supervision of the experiments, editing and finalizing of submitted manuscript. All authors have read and agreed to the published version of the manuscript.

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