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Sulfated Flavonoids in the Asteraceae

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Abstract:

Gypsum soils are a unique soil type with high levels of calcium and sulfur, which creates a harsh living environment for plants. To survive these conditions, plants have evolved a suite of mechanisms to survive these excess minerals, such as sulfur. In this study we aimed to determine if gypsum status was related to sulfated flavonoid production in plants of the Asteraceae. Flower and leaf tissues were collected from herbarium specimens representing gypsophiles, gypsovags, and gypsofuges. These tissues were analyzed for the presence of sulfated flavonoids using Thin Layer Chromatography (TLC). We observed sulfated flavonoid production in leaf and flower tissues of many of our focal taxa. Within our data set patterns of sulfated flavonoid production were related to phylogeny, but not gypsum status. Future research should include a broader sampling of taxa to better elucidate these patterns. Further, our work suggests another potential mechanism for surviving on gypsum soils.

Introduction:

Gypsum soils (>60% $CaSO_4 \cdot 2H_2O$) are widely distributed across every continent yet remain understudied (Escudero, 2015). These soils are generally considered harsh environments for plant life, as their high calcium and sulfur levels can be toxic and can interfere with uptake of other nutrients (Palacio et al., 2007). Due to its high solubility, gypsum soils are exclusively found in arid and semi-arid locations where plants often must overcome drought

stress. Further, their high calcium and sulfur levels have created a selective force for evolution by natural selection, yielding plants with a suite of adaptations supporting their success on these soils (Hoffmann et al., 2000). As a result, gypsum soils often support biodiversity hotspots (Escudero et al., 2015), with a suite of gypsophiles (well-adapted taxa found only on gypsum soils) and gypsovags (which grow on and off gypsum).

In these sulfur-rich environments, plants must either exclude sulfur or sequester it in some way. Unlike gypsophiles from younger lineages or gypsovags, which typically maintain lower leaf S, widely distributed gypsophiles in both Spain and the U.S.A. have independently evolved to uptake high amounts of sulfur into their leaves, with this sulfur present primarily in assimilated forms (Palacio et al., 2007). The incorporation of sulfur into compounds may function as a sulfur sink, decreasing the toxicity of the sulfur ion. This pattern has been documented in other systems where the production of secondary metabolites can be an adaptation to a new environment or can be used as a defense mechanism against pathogens or predators (Yanna et al., 2018). Some plants use the accumulation of gypsum crystals as a way to prevent sulfur toxicity (Robson et al., 2017). Other plants, namely those in the mustard family, are hypothesized to use the production of glucosinolates (a defense chemical) as a sink for excess sulfur (Borpatragohain et al., 2019).

Although not tested, the production of sulfated flavonoids could be another possible mechanism to prevent sulfur toxicity in gypsophiles. Flavonoids are polyphenolic compounds with a C6-C3-C6 structure, and sulfated flavonoids (Figure 1) have a sulfate group attached at the hydroxyl group (Kleinenkuhnen et al., 2019). These secondary metabolites are produced in a variety of plant tissues, and in total there are 56 known sulfated flavonoids, which have been

found in at least 250 species (Barron et al., 1988). These compounds have been detected in a variety of taxa in the Asteraceae (Barron et al., 1988), a family common among gypsophilic communities (Mandujano et al., 2020). However, many genera in the Asteraceae remain untested for sulfated flavonoids, and their presence among gypsophilic taxa is unknown. Testing for presence of sulfated flavonoids in gypsophilic asters could provide valuable insight into how plants survive in the harsh conditions posed by gypsum soils. This study aims to determine if plants in the Asteraceae growing on gypsum produce sulfated flavonoids. We hypothesize that plants in the Asteraceae do produce sulfated flavonoids in both their leaves and flowers, and that gypsophiles are more likely to produce sulfated flavonoids than gypsovags or gypsofuges (plants not growing on gypsum) because they are better adapted to the gypsum environment.

Methods:

Leaf and flower tissue samples were collected from plants in the Asteraceae growing on and off gypsum soils in New Mexico, Texas, and Mexico. The tissues were originally used for herbarium voucher specimens (Table 1) by Dr. Michael Moore and small samples were collected from the Oberlin College herbarium for this project in early 2020. The samples were stored in plastic centrifuge test tubes at ambient temperature in a laboratory area with no light exposure until use.

Thin Layer Chromatography (TLC) (Figure 2) was used to test for the presence of sulfated flavonoids. This method separates compounds in a mixture and allows for determining the presence/absence of compounds of interest, in this case, sulfated flavonoids (Desrochers,

1993). *Lasthenia californica* was used as the laboratory standard, as it is known to produce these compounds (Rajakaruna et al., 2003). Approximately 0.5mg of tissue was combined with five drops of 100% methanol (added one drop at a time until the tissue was completely saturated) and allowed to sit for 24 hours. The samples were then spotted onto Polyamid 6.6 plates (Sigma Aldrich) with a capillary tube, and the plates were placed in water-n-butanol-acetone-dioxane (70:15:10:5) until the solvent ran up the plate. After the plate was dry it was sprayed with 1% amino-ethyl-diphenylborate, a fixative that helps prevent the banding pattern from breaking down or fading. Finally, the plate was examined under a 366 nm UV light to observe the banding pattern. Sulfated flavonoids were scored as present in the sample based on the presence of a bright yellow band as detected in the laboratory standard.

The collected data was analyzed in R version 4.0.2. using logistic regression (similar to Litwinowicz, 2021). The flower and leaf data were analyzed separately. However, in both cases the predictor variable was gypsum status (gypsophile, gypsovag, gypsofuge) and the response variable was the presence/absence of a sulfated flavonoid band.

Results:

Based on results of logistic regression, gypsum status was not a significant predictor of presence or absence of sulfated flavonoids ($P > 0.05$ for gypsofuges and gypsophiles, respectively). Therefore, when compared to the gypsum status of gypsovag, neither gypsofuges nor gypsophiles were more likely to produce sulfated flavonoids in their leaves (Figure 3A).

Similarly, the flower sample results reflected the trends seen in the leaf sample results, with gypsum status not significantly predicting presence or absence of sulfated flavonoids in

flowers ($P > 0.05$ for gypsofuges and gypsophiles, respectively) (Figure 3B). Regardless of gypsum status, plants were not more likely than gypsovags to produce sulfated flavonoids in their leaves.

However, there do appear to be some trends within the presence/absence patterns of sulfated flavonoid production related to phylogeny (Figure 4). More closely related species seem to exhibit similar patterns of sulfated flavonoid production. *Coreopsis tinctoria* and *Coreopsis sp.* are closely related to one another and only exhibit sulfated flavonoids in their flower tissue. Similarly, *Haploethes greggii, Sartwellia flaveriae, Flaveria oppositifolia,* and *Flaveria chlorifolia* are all closely related and produce sulfated flavonoids in their flowers. Two of the most closely related taxa we used are *Flaveria oppositifolia,* and *Flaveria chorifolia,* which both exhibited sulfated flavonoid production in their flowers and leaves. Overall, it appears that closely related taxa are likely to exhibit similar patterns of sulfated flavonoid production.

Discussion:

Some species in the Asteraceae did produce sulfated flavonoids and others did not; based on our analysis, this pattern was not significantly related to gypsum status. However, there does appear to be a trend within this family related to the production of sulfated flavonoids based on phylogenetic relationships. More closely related species exhibit similar patterns of sulfated flavonoid production (production in only flowers or only leaves, both flowers and leaves, or no production). These data indicated that evolutionary history may be a better predictor of sulfated flavonoid production than soil environment.

There were many limitations in this study that prevented us from obtaining more concrete results. The band labeled as the sulfated flavonoid band during the TLC method did not always perfectly match up with the lab standard. This observation is likely attributed to there being other compounds that did not separate from the sulfated flavonoid. Ideally, this bright band would be sampled from the plate and further separated via High-Pressure Liquid Chromatography (HPLC) and Liquid-Chromatography Mass Spectrometry (LC MS). However, we did not have access to the equipment necessary to perform that task. As a result, interpretating the presence/absence of the sulfated flavonoid band and determining which samples had one present was less conclusive in some samples relative to others.

Another limitation we faced was the inability to replicate samples. We had very limited amounts of plant tissue, which prevented us from having a large sample size and having several replicates per taxon. Increasing the sample size would have helped us to ensure our results were a more accurate representation of the sample. Further, a broader sampling of taxa may have helped us better resolve patterns based on gypsum status and phylogeny.

Overall, this project helped to improve our understanding of sulfated flavonoid production in the Asteraceae. However, further research should be done to explore these findings with larger sample sizes and procedures that can better separate the compounds in these tissues. The types of sulfated flavonoids present could be separated to see if closely related species or species grown in similar environments give rise to the same types of flavonoids. This would also allow for quantification of the flavonoids present which could discover new patterns within the Asteraceae. This project helped to lay the foundation for

future research and also helped to elucidate some patterns of sulfated flavonoid production within the Asteraceae.

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References:

Escudero, A., Palacio, S., Maestre, F., & Luzuriaga, A. (2015). Plant life on gypsum: a review of its multiple facets. *Biological Reviews*, 90(1), 1.

Palacio, S., Escudero, A., Montserrat-Martí, G., Maestro, M., Milla, R., & Albert, M. (2007). Plants Living on Gypsum: Beyond the Specialist Model, *Annals of Botany*, 99(2), 333-343.

Hoffmann, A., & Hercus, M. (2000), Environmental Stress as an Evolutionary Force, *BioScience*, 50(3), 217-226.

Yanna C., Teles, M., Souza, S., & de Fatima Vanderlei de Souza, M. (2018). Sulphated Flavonoids: Biosynthesis, Structures, and Biological Activities. *Molecules,* 23(2), 480.

Robson, T., Stevens, J., Dixon, K., & Reid, N. (2017). Sulfur accumulation in gypsum-forming thiophores has its roots firmly in calcium, *Environmental and Experimental Botany,* 137, 208-219.

Borpatragohain, P., Rose, T., Liu, L., Barkla, B., Raymond, C., & King, G. (2019) Remobilization and fate of sulphur in mustard, *Annals of Botany*, 124(3), 471–480.

Kleinenkuhnen N., Büchel F., Gerlich S., Kopriva S., & Metzger S. (2019) A Novel Method for Identification and Quantification of Sulfated Flavonoids in Plants by Neutral Loss Scan Mass Spectrometry, *Frontiers in plant science,* 10, 885.

Barron, D., Varin, L., Ibrahim, R., Harborne, J., & Williams, C. (1988). Sulphated flavonoids- an update. *Phytochemistry*, 27(8), 2375-2395.

Mandujano, M., et al. (2020), Plant Diversity and Ecology in the Chihuahuan Desert: Emphasis on the Cuatro ciénegas Basin, *Springer Nature Switzerland AG*.

Desrochers, A., & Bohm, B. (1993). Flavonoid variation in Lasthenia California (Asteraceae). *Biochemical Systematics and Ecology*, 21(4), 449-453.

Rajakaruna, N., Baldwin, B., Chan, R., Desrochers, A., Bohm, B., & Whitton, J. (2003). Edaphic races and phylogenetic taxa in the Lasthenia californica complex (Asteraceae: Heliantheae): an hypothesis of parallel evolution. *Molecular Ecology,* 12(6), 1675-1679.

Litwinowicz, Z. (2021). Heat and Chemical Cues Enhance Germination Rates in Fire-Dependent Chaparral Species, *Senior Honors Projects*, 125.

Tables:

Taxon	Collection number	Abbreviation	Status	Sampling site (lat/long)
Sartwellia flaveriae A.Gray	M.J. Moore 486	SAFL	wide gypsophile	31 °11 41.7, -105 31 38.5
Sartwellia flaveriae A.Gray	M.J. Moore 699	SAFL	wide gypsophile	34 36 04.1, -105 54 49.6
Haploesthes greggii A.Gray	M.J. Moore 391	HAGR	wide gypsophile	33 45 33.5, -106 07 00.7
Haploesthes greggii A.Gray	M.J. Moore 683	HAGR	wide gypsophile	34 48 01.6, -104 38 01.4
Haploesthes greggii A.Gray	M.J. Moore 434	HAGR	wide gypsophile	29 34 38.4, -103 36 32.5
Haploesthes greggii A.Gray	M.J. Moore 464	HAGR	wide gypsophile	29 18 50.5, -103 39 51.8
Haploesthes greggii A.Gray	M.J. Moore 586	HAGR	wide gypsophile	30 03 40.3, -102 17 27.3
Dicranocarpus parviflorus A.Gray	M.J. Moore 730	DIPA	wide gypsophile	32 39 59.7, -106 00 01.3
Dicranocarpus parviflorus A.Gray	M.J. Moore 909	DIPA	wide gypsophile	31 55 24.8, -105 03 57.1
Dicranocarpus parviflorus A.Gray	M.J. Moore 1930	DIPA	wide gypsophile	25 54 01.2, -101 39 10.0
Dicranocarpus parviflorus A.Gray	M.J. Moore 1991	DIPA	wide gypsophile	26 36 54.3,-103 01 32.5
Verbesina potosina L.	M.J. Moore 1834	VEPO	wide gypsophile	22 11 22.1. -100 20 56.0
Verbesina hintonioium L.	M.J. Moore 2828	VEHI	wide gypsophile	24 53 07.8, -100 11 28.0
Thelesperma simplicifolium A.Gray	M.J. Moore 1382	THSI	gypsovag	24 08 32.9, -99 51 07.8
Thelesperma megapotanicum Spreng.	M.J. Moore 1723	THME	gypsovag	29 54 33.4, -101 45 38.2
Flaveria oppositifolia A.M.Powell	M.J. Moore1284	FLOP	gypsovag	24 07 46.8, -100 22 46.1
Flaveria oppositifolia A.M.Powell	M.J. Moore 1295	FLOP	gypsovag	24 34 34.5, -100 15 52.6
Flaveria chlorifolia A.Gray	M.J. Moore 684	FLCH	gypsovag	34 56 21.8, -104 40 26.8
Gaillardia pinnatifida Torr.	M.J. Moore 128	GAPI	gypsovag	29 35 24.3, -103 33 36.9
Gaillardia pinnatifida Torr.	M.J. Moore 595	GAPI	gypsovag	30 22 30.06, -103 45 48.19
Gaillardia pinnatifida Torr.	M.J. Moore 2985	GAPI	gypsovag	33 14 03.0, -100 05 51.5
Brickellia lemmonii Elliot	M.J. Moore 2830	BRLE	gypsovag	24 53 07.8, -100 11 28.0
Coreopsis tinctoria Nutt	M.J. Moore 1659	COTI	not on gypsum	30 09 50.3, -97 48 50.8
Coreopsis tinctoria Nutt	M.J. Moore 1688	COTI	not on gypsum	27 19 13.1,-97 41 09.8
Coreopsis sp. Nutt	M.J. Moore 956	COSP	not on gypsum	29 14 05.3, -97 32 50.1
Brickellia eupatorioides L.	M.J. Moore 528	BREU	not on gypsum	40 05 58.36, -88 13 55.76
Verbesina virginica L.	M.J. Moore 548	VEVI	not on gypsum	30 09 11.70, -97 48 51.98

Table 1. Sample information for the herbarium specimens used in this study, including *Sartwellia flaveriae, Haploesthes greggii*, *Flaveria oppositifolia*, *Flaveria chlorifolia*, *Dicranocarpus parviflorus*, *Coreopsis tinctoria*, *Coreopsis sp.*, *Gaillardia pinnatifida*, *Brickellia*

lemmonii, *Verbesina potosina*, *Verbesina hintonioium*, *Thelesperma simplicifolium*, *Thelesperma megapotanicum*, *Brickellia eupatorioides,* and *Verbesina virginica.*

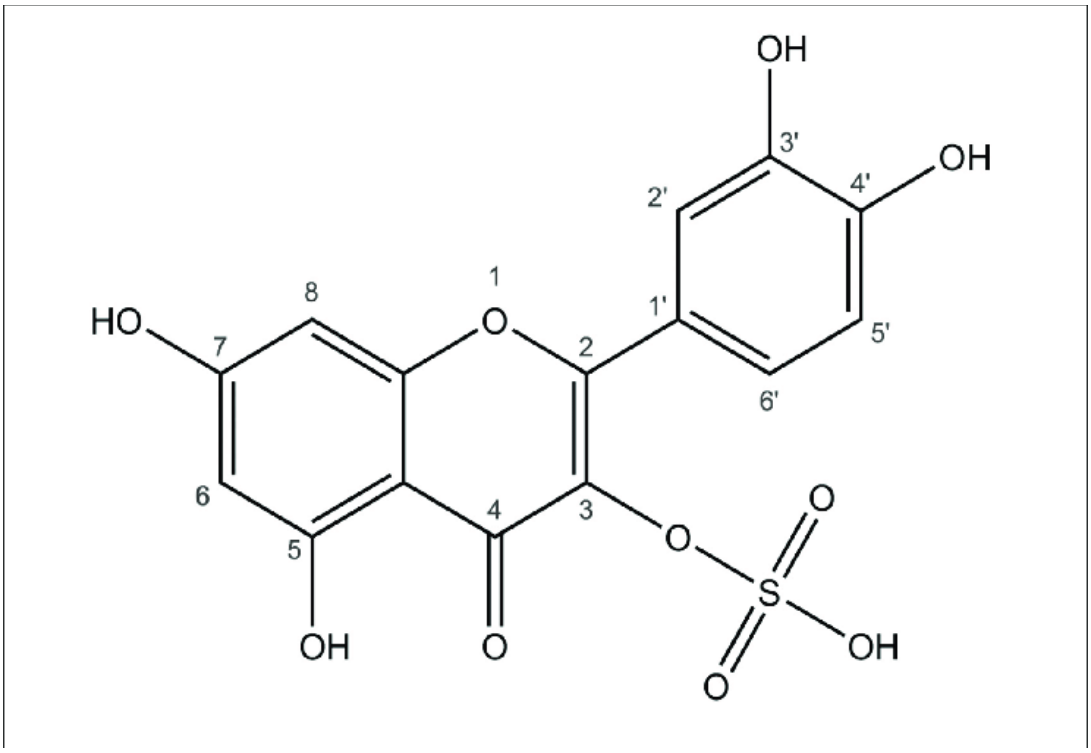
Figures:

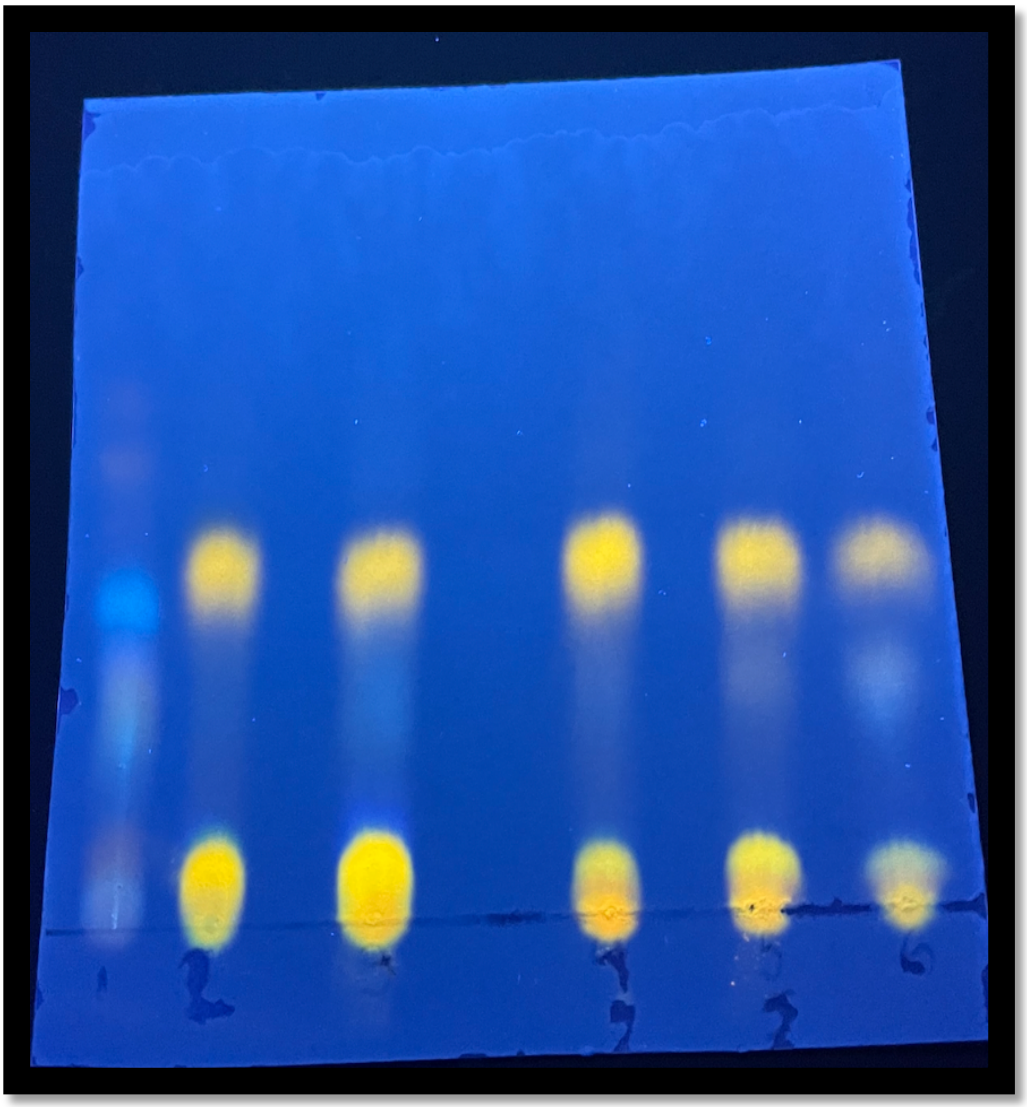
Figure 1. This figure represents the basic structure of a sulfated flavonoid (Kleinenkuhnen et al., 2019).

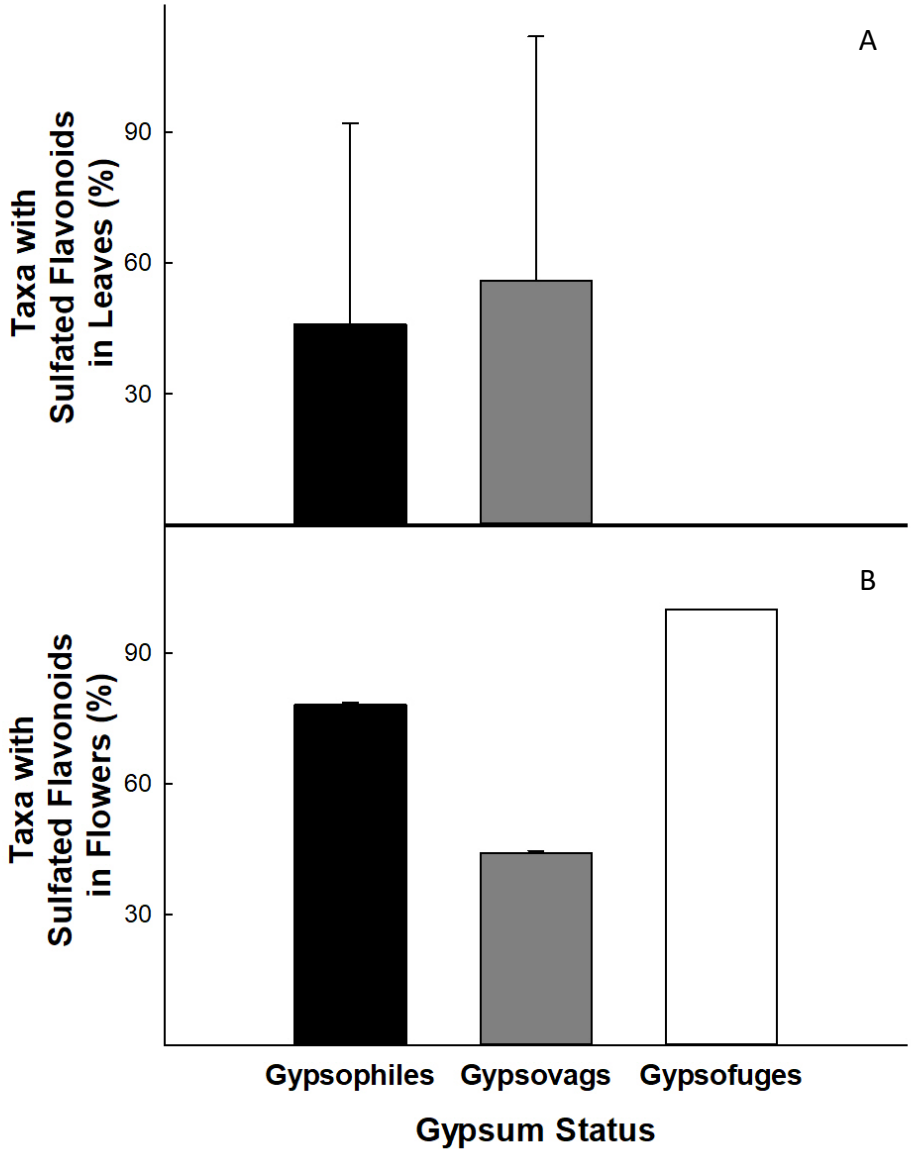
Figure 2. Representative TLC plate used in this study with the top yellow band indicating the presence of sulfated flavonoids.

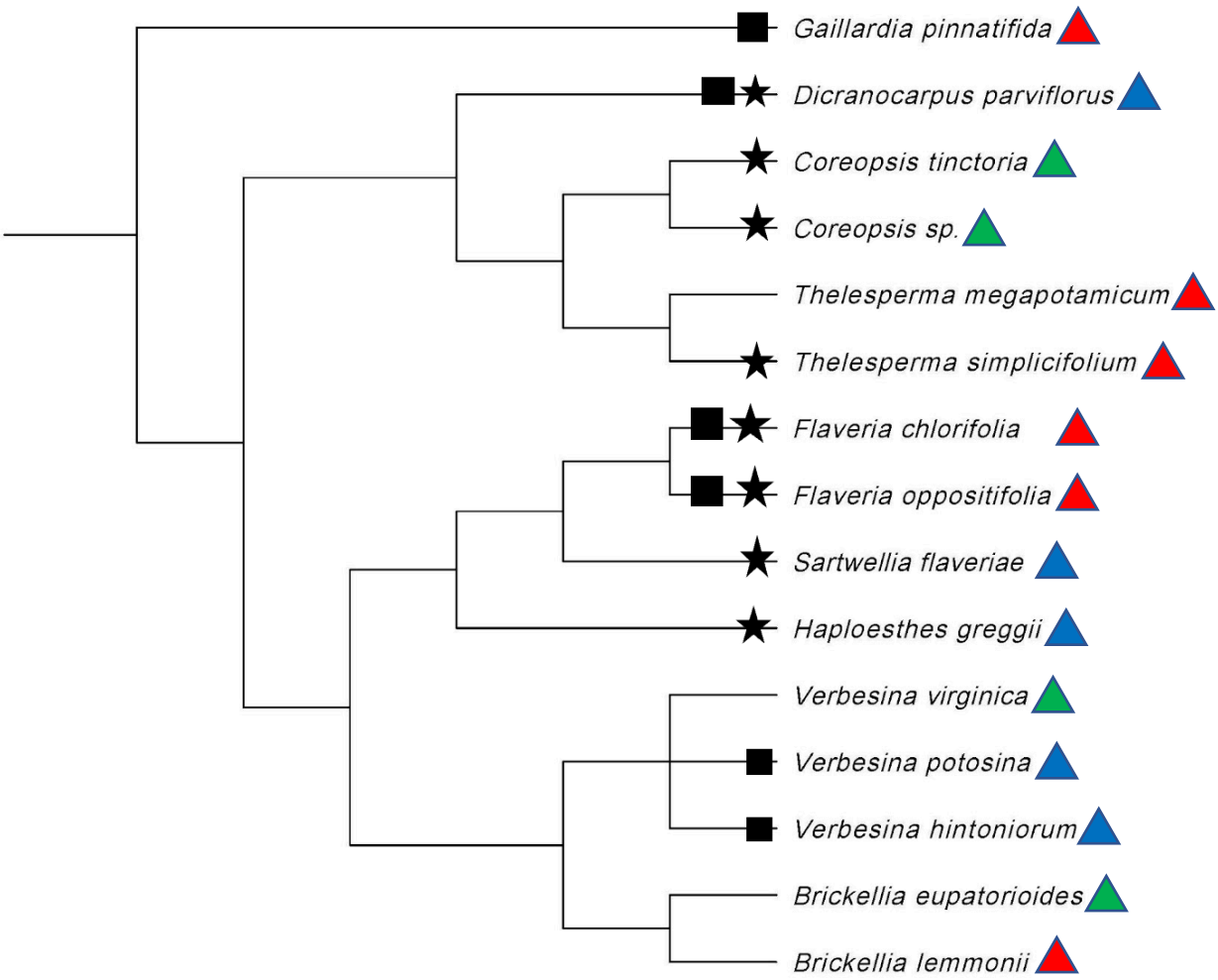
Figure 3. Taxa that produce sulfated flavonoids (%) in leaves (A) and flower (B). Data are means + SD (n=27 (A) and n=25 (B)).

Figure 4. This figure represents the phylogenic tree for the taxa used in this study (star indicates detection of sulfated flavonoids in flowers and square indicates detection of sulfated flavonoids in leaves. Red indicates gypsovag, blue indicates gypsophile, and green indicates gypsofuge).


OH
3'
OH
4'
2'
1
8
HO
7
O
2
1'
5'
6'
6
4
3
O
O
5
S
OH
OH
O
O




A
Taxa with Sulfated Flavonoids in Leaves (%)
90
60
30
B
Taxa with Sulfated Flavonoids in Flowers (%)
90
60
30
Gypsophiles
Gypsovags
Gypsofuges
Gypsum Status


Gaillardia pinnatifida
Dicranocarpus parviflorus
Coreopsis tinctoria
Coreopsis sp.
Thelesperma megapotamicum
Thelesperma simplicifolium
Flaveria chlorifolia
Flaveria oppositifolia
Sartwellia flaveriae
Haploesthes greggii
Verbesina virginica
Verbesina potosina
Verbesina hintoniorum
Brickellia eupatorioides
Brickellia lemmonii