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Total phenolic and flavonoid contents of *Elephantopus scaber* and *Ageratum conyzoides* (Asteraceae) leaves extracts from various altitude habitats

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ABSTRACT

Asteraceae family has various benefit as herbal medicine and phytochemical affect. It can grow in different habitats but secondary metabolites in Asteraceae depend on the environmental factors. The aims of the study were to quantitatively analyze the phenolic and flavonoid content of two kinds of plants from Asteraceae family, such as *Elephantopus scaber and Ageratum conyzoides*, on three differents kinds of habitats which differ on the altitudes. *Elephantopus scaber* and *Ageratum conyzoides* leaves were obtained from three different altitude habitat: lowland (Bangkalan-Madura; 28.3 - 31.72 m asl), middle land (Trawas- Mojokerto; 727 – 937 m asl) and highland (Coban talun-Batu; 1303 – 1322 m asl). The simplicia of Asteraceae family leaves were macerated and extracted with methanol, ethyl acetate, aquades and n-butanol. The total phenolic (gallic acid/GAE) and flavonoid (quercetin/QE) contents were determined using UV-VIS spectrometer. The results were then analyzed by ANOVA. The results showed that the total phenolic (1.86 ±0.03 mg/mL) and flavonoid (3.4 ±0.06 mg/mL) contents of *Elephantopus scaber* were showed in the middle-altitude land was found higher as compared to the lowland (total phenolic content 1.566±0.04 and flavonoid 3.2±0.12 mg/mL) and highland (total phenolic content 1.417±0.04 and flavonoid 3.1±0.01 mg/mL). The total phenolic contents of *A.conyzoides* in middle-altitude (1.66±0.1 mg/mL) was higher than highland (1.30±0.03 mg/mL) and lowland (1.25±0.02 mg/mL). The total flavonoid on *A.conyzoides* in highland (3.2±0.06 mg/mL) was higher than *A. conyzoides* growing in the middle-altitude land (2.9±0.0 mg/mL) and in lowland (2.6±0.06 mg/mL). The highest phenolic content was found to be in methanol extract, and the highest flavonoid content was found to be in ethyl acetate fraction of *Elephantopus scaber* and *A. conyzoides.*

Key words : *Elephantopus scaber, Ageratum conyzoides, Phenol, Flavonoid, Leaves extracts, Altitude habitat*

Introduction

In metabolism, plants produce not only primary compound in metabolism, but also secondary metabolites. Those are phenolic compounds, alkaloids, terpenoids, and sulfur compounds. These secondary metabolites are a defense mechanism against pests (Lambers *et al*., 1998). Asteraceae is a family of plant that can be found widely, with over 20,000 species and more than 1100 genera (Cronquist, 1997). Most

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of Asteraceae members contain chemical compounds such as polyphenols, flavonoids, and sesquiterpene. Asteraceae has a broad diversity of phytochemicals, including pyrethrum, triterpenoids, saponins, coumarin and flavonoids (Ozgen *et al*., 2004). Research on phenol compounds has been conducted on various plants in the Asteraceae family. The results showed that phenolic compounds can be found in many Asteraceae members and its contents are quite large, so it can be used as an indicator of secondary metabolites present in Asteraceae that are located in different habitats.

Researches on Asteraceae members, namely *Artemisia austriaca, Achillea biebersteinii,* and *Helichrysum arenarium* which were extracted using methanol, resulted that: *Artemisia austriaca* contained flavonoid and sesquiterpen lactone, *Achillea biebersteinii* contained flavonoid, *Helichrysum arenarium* contained flavonoid and simple phenolic compound. Research show that methanol extract of *Achillea biebersteinii* describe antioxidant activity. Overall plants indicate the presence of phenolic compounds, especially flavonoids (Ozgen *et al.*, 2004). Research on *Anaphalis contorta* Hook. f. was identified to contain phenolic compounds: syringic acid, vanillic acid, p-hydroxybenzoic acid, ferulic acid, protocatechuic acid, gallic acid, p-coumaric acid and 3,5-dihidroxybenz1oic acid. Phenolic compounds act as antioxidant, anti-aging, anti-inflammation, and inhibit cell proliferation activity (Joshi, 2011).

Phenol compounds of *Rhagadiolus stellatus* (Asteraceae, Cichorieae) had been investigated by Krimplstatter *et al.* (2011) and the results showed that these plants contain flavonoid kaempferol 3-O-β-glucoside, kaempferol 3-O-β¬rutinoside (nicotic florin), quercetin 3-O-β-glucoside, and luteolin. Riedel *et al*. (2010) conducted a study of phenolic compounds from *Artemisia frigida* and *Sylybum marianum*, the results showed that *Sylybum marianum* contained a total phenol 255.8 µmol/g dry weight, while *Artemisia frigida* contained 173.9 µmol/g dry weight. Total phenolic acid content was seen from vanillate, chlorogenic acid, caffeic acid, coumarin acid, ferulic acid, cinnamic acid, it is also said that the phenolic compounds act as an attractant, repellant and protection of plants against insects, fungi, bacteria and viruses. Research on *Crassocephalum crepidioides* (Benth.) S. Moore. using ethanol extract was analyzed with phenol contained at 422.22 mg/g (gallic acid) and flavonoids at 3.46 mg/g (quercetin) (Wijaya, 2011). Research on *Hieracium pilosella* L. indicated that the plant phenolic compounds contained a total of 239.59 to 244.16 mg/g (chlorogenic acid) and flavonoids from 79.13 to 82.18 mg/g (Stanojevic *et al.,* 2009). *Ageratum conyzoides*, and *Elephantopus scaber* are members of Asteraceae which known to contain active ingredients so that people use those plants as medicine and natural pesticide.

Elephantopus scaber grows wild and can be found from the lowlands to an altitude of 1200 m above sea level (asl). *E. scaber* leaf has medicinal properties and produces secondary metabolites containing alkaloids, tannins, phenols, proteins, glycosides, saponins, terpenoids and steroids. It also contains epifriedelinol, lupeol, stigmasterol, triacontan-1-ol, dotria-contan-1-ol, lupeol acetate, deoxyelephantopin, isodeoxyelephantopin, and luteolin-7-glucosida, which act as antimicrobial (Wan Yong Ho *et al*., 2009; Yuliani *et al.,* 2018).

Ageratum conyzoides is a plant used to control locusts (Grainge and Ahmed, 1988). It contains secondary metabolites of saponins, polyphenols, coumarin, eugenol, essential oils, alkaloids, tannins, and sulfur. N-hexane extract and methanol extract of *Ageratum* leaf showed antifungal activity, antibacterial and ability to inhibit insect juvenile hormone (Ming, 1999). Renuga and Sahayaraj (2009) in their study to see the effect of the ethanol extract of the genus *Ageratum* concluded that extract of *Ageratum conyzoides* and *Ageratum vulgaris* can significantly lower than the total protein of *Spodoptera litura* at a concentration of 0.01 µL/insects.

Plant secondary metabolites are formed in an attempt to defend themselves from the ecosystem. The content of secondary metabolites in plants is affected by the environment such as altitude, rainfall, and temperature (Vanhaelen *et al.,* 1991). Furthermore, he said that the influence of environmental factors interact with genetic factors in the expression of secondary metabolites, so that the production and excretion of secondary metabolites are affected by temperature, light, soil, microorganisms, and nutrient status (Olofsdotter, 2001). Inderjit and Keating (2003) reported the content of secondary metabolites (allelochemicals) varies from one location to another and from time to time. Content variations are related to variations in climate and soil conditions such as air and soil temperature and soil moisture. It was also explained by Mazid *et al.* (2011) that stress caused by biotic and abiotic factors

affected the production of secondary metabolites, and generally tended to increase the production of secondary metabolites. Therefore, if the plants are cultivated, it must be adapted to the habitat so that the content of secondary metabolites can be produced optimally. Information about the potential diversity of secondary metabolites is a consideration in plant cultivation.

Based on above reasons, this study aims to measure the content of phenolic compounds in plants of the Asteraceae (*Ageratum conyzoides* and *Elephantopus scaber*) in various habitats (altitude), those are in the lowlands (3-50 m asl), middle-altitude land (700-900 m asl) and highland (>1300 m asl). Family Asteraceae was chosen because it generally contains phenolic compounds such as coumarin, flavonoids and tannins, easy to grow in all places from the lowlands to the highlands (up to 1500 m asl) and easily cultivated, so the range of spread is very wide. In addition, *Elephantopus scaber* and *Ageratum conyzoides* are usually used as herbal medicine or natural pesticides.

Materials and Methods

Plant material used

Sampling sites (the leaves of *Elephantopus scaber*, and *Ageratum conyzoides*) were selected by purposive sampling method in lowland < 50 m asl, 700-950 m asl for middle-altitude land, and > 1300 m asl for highland. They were Bangkalan (Madura) for lowland (28.3-31.72 m asl), Dlundung - Trawas (Mojokerto) for middle-altitude land (727-937 m asl) and Coban Talun - Bumiaji (Batu Malang) for highland (1303-1322 m asl). The sampling method was conducted at three different areas showed in Fig. 1.

Extraction and fractionation method

Extraction and Fractionation process of *Elephantopus scaber and A.conyzoides* leaf using the procedure of Dorman and Hiltunen (2004). *Elephantopus scaber*, *A.conyzoides* plant leaf powder (40 mesh size) was macerated with petroleum ether at room temperature for 24 hours. The dried residue was extracted with methanol using soxhlet extraction at a temperature of 65 ^{o}C for 3 hours. Methanol solvent was evaporated with a rotary evaporator. The extract obtained was fractionated with ethyl acetate and distilled water. Next, distilled water phase was fractionated with n-butanol solvent. Solvents in fraction of ethyl acetate, n-butanol, and distilled water were evaporated with rotary evaporator, each extract and fractions were stored at 4 °C until next analysis.

Total phenolic and flavonoid contents

The total phenolic contents of *Ageratum conyzoides*, *Elephantopus scaber* extracts were determined using

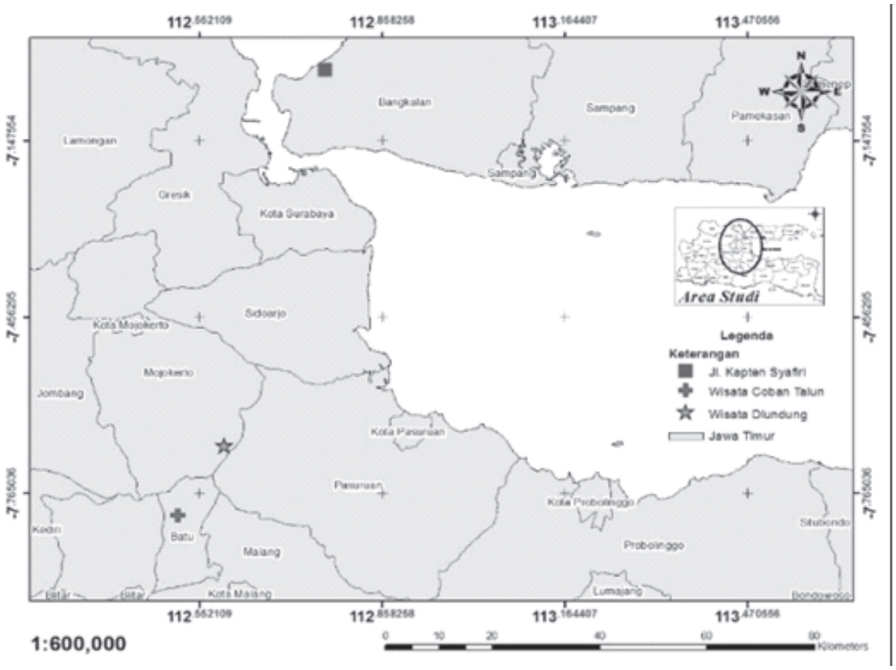

Fig. 1. Location map of three sampling sites in East Java

spectrometer according to Folin-Ciocalteu method (Singleton *et al.,* 1999), gallic acid was used as a standard (the concentration range 0.125 to 0.625 mg/mL). The total phenolic content was expressed as gallic acid equivalent (GAE) using calibration curve.The total flavonoid content was determined according to the aluminium chloride colorimetric method, as described by Kumar *et al*. (2008). The total flavonoid content was determined as quercetin equivalent (QE) using calibration curve. Parameter of this study are the contents of phenolic and flavonoid compounds in *E. scaber, A.conyzoides* obtained at three locations based on altitude difference, with three replications of extraction. Data were analyzed using ANOVA.

Results

Based on the ecological characters, *E. scaber* and *A.conyzoides* could grow in altitude from 28.7 meters to1312.2 meters asl, temperature from 27.57 °C to 38.63 °C, humidity from 59% to 83.5%, atmospheric oxygen level from 17.35% to 21.25% , and light intensity from 153.21 to 808.580 lux. Based on the chemical analysis of soil, *E. scaber* and *A.conyzoides* could live in the slightly low soil pH (middle-altitude land: 6.26) to neutral (lowland/highland were each 7.05 and 6.08), low organic C (lowland/1.18%), medium (middle-altitude land/2.7%) and high (highland/3.31%), low N nutrient (lowland/0.127%) to medium (middle-altitude land/0.272% and highland/0.335%). Based on soil physical properties, *E. scaber* and *A.conyzoides* can be grown on sandy clay-loam soil with soil porosity from 46.25 to 70.08 (% vol), and soil water level from 0.19 to 0.46 cm^3. Highlands have a higher level of organic materials, N nutrients, and water level than the lowlands and middle-altitude land.

Research data includes total phenol content and total flavonoid content of *A. conyzoides* and *Elephantopus scaber* and phenol and flavonoid content to various solvent and fractions.The results of the use of solvent extracts and fractions (methanol, ethyl acetate, butanol and water) to phenol and flavonoid mg/mL of Asteraceae plants can be seen in Table 1 and Table 2.

Analysis of variance result to different test of extract solvent (methanol, ethyl acetate, butanol and water fraction) to the phenols content showed that there are different content of gallic acid significantly among methanol, butanol, ethyl acetate and water fraction in *E. scaber* (F count 392,013 > F table 3,008) and *A. conyzoides* (F count 101,286 > F table 3,008). Based on Table 1, it is known that in the methanol extract had the highest phenols level, and lowest in water fraction.

Analysis of variance result to different test of extract solvent (methanol, ethyl acetate, butanol and water fraction) to the flavonoids level (Table 2), showed that there were different flavonoids level significantly between methanol, butanol, ethyl acetate and water fraction (F count 37,533> F table 3,008) in *E. scaber*, and *A. conyzoides* (F count 72,333> F tables 3.008). Ethyl acetate fraction had the highest flavonoids level, followed by methanol extract, butanol fraction and the lowest for the water fraction. Based on the level of phenolic compounds obtained, methanol extract was used for the determination of plant compounds, and will also be used to test the bioactivity.

The results of the study on total phenols and total flavonoids level at all two Asteraceae plants in different habitats (Table 3) indicated that *E. scaber* showed the highest phenol contained in *E.scaber*

Table 1. The solvent extracts and fractions (methanol, ethyl acetate, butanol and water) to phenols level mg/mL in *Elephantopus scaber* and *Ageratum conyzoides*

Plant name/Altitude	Phenols level mg/mL using solvent			
Habitat	Methanol Extract	Ethyl Acetate Fraction	Butanol Fraction	Water Fraction
E. scaber				
Lowland	0.570±0.027	0.370±0.024	0.471±0.011	0.156±0.023
Middle-altitude land	0.611±0.009	0.536±0.003	0.495±0.032	0.220±0.020
Highland	0.435±0.034	0.497±0.003	0.322±0.032	0.163±0.030
A. conyzoides				
Lowland	0.518±0.058	0.366±0.037	0.180±0.022	0.193±0.017
Middle-altitude land	0.536±0.067	0.445±0.024	0.417±0.019	0.264±0.059
Highland	0.432±0.013	0.382±0.005	0.249±0.019	0.239±0.009

that grows in the middle-altitude land, followed by *E.scaber* in the lowlands, and the lowest is in the highlands, while the highest levels of flavonoids found in *E. scaber* in the middle-altitude land, while for the highlands and lowlands did not show any difference. *A. conyzoides* showed that the highest phenol level contained in *A. conyzoides* that grow in the middle-altitude land, while for the lowland and highland showed no difference, while the highest levels of flavonoids obtained at *A. conyzoides* that grows in the highlands, followed by middle-altitude land, and lowlands.

Results of analysis of variance indicated (Table 3) that there are significant differences between the phenol level of *E. scaber* (F count 23.558> F tables 3.402), and *A. conyzoides* (F count 29.826> F tables 2.508) in the lowland areas, middle-altitude land and highlands. Phenol levels in the two Asteraceae plants differed significantly (significance value less than 0.05). Similarly, levels of phenol in the third altitude habitats also differed significantly (<0.05). There is a significant interaction between plant species and habitat altitude on levels of total phenols. Plant species that have the maximum phenol levels are plants *E. scaber*, followed *by A. conyzoides.*

Results of analysis of variance showed that there are significant differences between the levels of flavonoids in *E. scaber* growing in various altitude habitat (F count 16.800> F table 2.508), and also there are different levels of flavonoids in *A. conyzoides* in the lowlands, middle-altitude land and highlands (F count 81.333> F tables 2.508). There is a significant interaction between plant species and habitat altitude on flavonoids level. Plant species that have the maximum levels of flavonoids was *E. scaber*.

Based on the pattern of spatial variation relationship with all two plants to levels of phenols and flavonoids, we can conclude for the cultivation of: 1) *A. conyzoides*, the lower the area of growing, level of phenol and flavonoid also low, and the higher the area of growing, the levels of phenols and flavonoids produced were also higher, but the plant will have the maximum levels of compounds in middle-altitude lands (neither too low nor too high), 2) *E. scaber*, the higher the area of growth, the levels of phenols and flavonoids produced were lower. Therefore, this plant will have a maximum content of phenols and flavonoids in middle-altitude lands (neither too low nor too high). Thus, the proper location to cultivate the two plants based on phenol and flavonoid, are 1) *E. scaber* planted in the middle-altitude lands (Trawas), and 2) *A. conyzoides* planted in middle-altitude lands (Trawas). From

Table 2. The solvent extracts and fractions (methanol, ethyl acetate, butanol and water) to flavonoids level mg/mL in *Elephantopus scaber* and *Ageratum cony*

Plant name/Altitude Habitat	Flavonoids level mg/mL using solvent			
	Methanol Extract	Ethyl Acetate Fraction	Butanol Fraction	Water Fraction
E. scaber				
Lowland	0.93±0.057	0.83±0.057	0.80±0.000	0.60±0.000
Middle-altitude land	0.83±0.057	0.92±0.000	0.90±0.000	0.80±0.000
Highland	0.87±0.057	0.72±0.057	0.80±0.000	0.70±0.000
A. conyzoides				
Lowland	0.50±0.000	0.73±0.057	0.70±0.000	0.63±0.057
Middle-altitude land	0.70±0.000	0.80±0.000	0.80±0.000	0.60±0.000
Highland	0.80±0.000	0.80±0.000	0.90±0.000	0.67±0.057

Table 3. Total phenol and flavonoid levels (mg/mL) of Asteraceae in various habitat

Plant name	Altitude habitat	Phenol (mg/mL)	Flavonoid (mg/mL)
A. conyzoides	Lowland	1.257±0.02 b	2.6±0.06 c
	Middle-altitude land	1.662±0.10 a	2.9±0.00 b
	Highland	1.302±0.03 b	3.2±0.06 a
E. scaber	Lowland	1.566±0.04 b	3.2±0.12 b
	Middle-altitude land	1.861±0.03 a	3.4±0.06 a
	Highland	1.417±0.04 c	3.1±0.01 b

Asteraceae plants, *Elephantopus scaber* cultivated in middle-altitude lands had higher levels of phenolic compounds compared to the *A. conyzoides.*

Discussion

The potential of secondary metabolites (production, persistence, and effectiveness) of an organism and its effects on the target organisms had diversity generally caused by genetic or environmental factors. The influence of environmental factors needs to be emphasized because of their interaction with genetic factors in the phenotypic expression of secondary metabolites. The diversity of secondary metabolites in plants is caused by interaction between plants with ever-changing environment produced a variety of compounds to defend them self against the abiotic and biotic environment. Secondary metabolites are the result of the metabolic adaptation of plants to the environment. Plant secondary metabolites protect against a variety of herbivores and pathogens and various abiotic stresses (Mazid *et al*., 2011). Similarly, in this study, although all three plants were from Asteraceae, but they were genetically different resulting in phenotypic expression of different secondary metabolites, in this case *E. scaber* had the largest content of phenolic compounds followed by *A. conyzoides*.

One way of biosynthesis of phenol is catalyzed by phenylalanine ammonia lyase (PAL). PAL is the branching between primary and secondary metabolism, so its catalyzed reaction is an important stage in the formation of many phenolic compounds. PAL activity can be increased due to several factors, such as low levels of nutrients, light (through its effect on photochrome), and microbial infections. Control point located on the initiation of transcription. PAL activity regulation in plants is maintained by various PAL coding genes in many species, and some expressed only in certain tissues or under certain environmental conditions (Taiz and Zeiger, 2010). Most enzymes such as PAL is induced by stress and play an important role in plant protection. PAL along with cinnamate 4-hydroxylase is an important group of enzymes in allocating large amounts of carbon from phenyl alanine into the biosynthesis of some important secondary metabolites (Khan *et al*., 2011). In general, when the plant is stressed, the production of secondary metabolites can be increased because the growth is often inhibited because fixed carbon is not allocated to growth, but allocated to the formation of secondary metabolites (Mooney *et al*., 1991; Gairola *et al*., 2010).

The effect of altitude habitat to the level of secondary metabolites was demonstrated by the following study, quercetin is found on processed soil but is not found in soil that is not cultivated. The higher the solar radiation has an impact on secondary metabolite profiles. For example the production of phenolic compounds increased as a response to increasing UV radiation (Jaakola and Anja, 2010). Research on *Hypericum perforatum* L. growing under different temperature and light intensities indicated that the accumulation of secondary metabolites is very dependent on temperature, light intensity and phenological cycle. The total of phenolic compounds greatly changed during the development phase of the plant, and the highest level achieved in the flowering phase with the higher light intensity and temperature (Radušienë, *et al*., 2012). Increased ozone (average 32.4 ppb) can increase the total phenol content of the leaves (Savirnata *et al*., 2010).

Variations of secondary metabolites were related to variations in climate and soil conditions such as air and soil temperature, soil moisture, acidity, organic C and nutrient content, so the altitude habitat that affected environmental conditions (climate and soil) would also affect the diversity of secondary metabolites produced. Described by Mazid *et al.* (2011), environmental stress caused by biotic and abiotic factors, this would affect the production of secondary metabolites, and generally tends to increase the production of secondary metabolites. So that when the plant was under pressure (stress), there is an exchange between carbon for biomass production or for the formation of secondary metabolites of defense. A stress response induced when plants are under stress at the cellular level. Secondary metabolites are involved in the protection of plants, in response to biotic and abiotic stress conditions (Ramakrishna *et al.,* 2011).

The existence of organic matter in the soil also supports secondary metabolites in plants. The expression of secondary metabolites in the field is influenced by soil texture, nutrients, pH, organic C, soil cultivation techniques and cropping systems. Nitrogen and phosphate deficiency directly affects the accumulation of phenylpropanoids. Potassium (K), Sulfur (S), Magnesium (Mg) and iron (Fe) deficiency can also increase the concentration of phenolic and increase the release of phenolic in nature (Junaedi *et al.,* 2006; Ramakrishna *et al*., 2011). This

is also supported also by soil texture data in the middle-alti tude lands which showed that the clay-textured soil had sufficient levels of organic matter and N nutrient, and C/N ratio 10 was close to C/N soil ratio. Good soil conditions resulted in optimal growth, and created large secondary metabolite levels in middle-altitude areas.

Conclusion

The total phenolic contents of *A.conyzoides* in middle-altitude (1.66±0.1 mg/mL) was higher than in the highland (1.30±0.03 mg/mL) and lowland (1.25±0.02mg/mL). The total flavonoid on *A.conyzoides* in highland (3.2±0.06 mg/ mL) was higher than *A.conyzoides* growing in the middle-altitude land (2.9±0.0 mg/ mL) and in lowland (2.6±0.06 mg/mL). The total phenolic (1.86 ±0.03 mg/mL) and flavonoid (3.4 ±0.06 mg/mL) contents of *Elephantopus scaber* were showed in the middle-altitude land was found higher as compared to the lowland and highland. The highest phenolic content was found to be in methanol extract, and the highest flavonoid content was found to be in ethyl acetate fraction of *Elephantopus scaber* and *A.conyzoides*.

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