THE NUTRITIVE VALUE AND BIOACTIVE COMPOUNDS OF ALFALFA (*Medicago sativa*) GROWN AT BURAPHA UNIVERSITY, SA KAEO CAMPUS

SUPREENA SRISAIKHAM, QUANJAI RUPITAK*

Faculty of Agricultural Technology, Burapha University, Sa Kaeo Campus, Sa Kaeo 27160, Thailand

*Corresponding author: quanjair@buu.ac.th

Abstract:

Legume is a good quality protein source that provides nutritive value for livestock animals, particularly ruminants. It also contains bioactive compounds which can be work against oxidants that prevent diseases. Alfalfa (*Medicago sativa*) is a perennial flowering plant in the legume family Fabaceae with a high protein content and moderate contents of vitamin B, C, phosphorus, and zinc. The aim of this research was to evaluate the nutritive value and bioactive compounds of alfalfa at harvesting intervals of 120 days at Sa Kaeo province. Alfalfa was grown at the agricultural research facility of the Faculty of Agricultural Technology, Burapha University, Sa Kaeo Campus, Thailand. They experiment was done from September 2018–March 2019. Chemical composition and bioactive compounds (tannin, total phenolic, total flavonoids, and isoflavone) were measured. The results showed that dry matter, ash, crude protein, fat and crude fiber were 91.44%, 9.20%, 17.68%, 2.46%, and 26.48%, respectively. Whereas, tannin, total phenolic, total flavonoids, daidzein and genistein in dry alfalfa were 2.34%, 2.17 mg RE/g sample, 2.02 mg RE/g sample, 46.72 and 15.38 µg/g sample, respectively. The highest phenolic compounds were 203.60 µg/g sample. The results suggest that the alfalfa plant can be developed as supplement that is benefit to health, for dairy cattle to achieve alternative products that contains high-flavonoids level in goat or dairy cow's milk that will be useful for ruminant and human health as well.

Keywords: Legumes; Alfalfa; *Medicago sativa;* Nutritive value; Bioactive compound

Introduction

Alfalfa (*Medicago sativa*) is the forage legume which is an important crop for livestock animals, particularly ruminants. It has been reported that many species of legumes have been used as the raw materials for animal feed, in the form of green feed, hay or pellets, especially in the tropics and sub-tropical regions. Legumes that have been used as fodder and roughage, such as alfalfa forage, hedge lucern (*Desmanthus virgatus*), hamata (*Stylosanthes hamata*), and Tha Phra Stylo (*Stylosanthes guianensis*) which are forage legumes that use parts of the stem, branches and leaves of plants as an animal feed sources, and in comparison to acacia or Leucaena foliage (*Leucaena leucocephala*) which is one of the fastest-growing leguminous trees. Legumes are resistant to frequent cutting and grazing with high palatability (Clement, 2019). Forage legumes are also high voluntary intake and ruminant animal production when feed supply is non-limiting compared to grasses or cereals (Phelan *et al.,* 2015). In addition, legumes leaf contained both high nutrients and protein leaf that can be used in the diet of ruminant animals. However, *Desmanthus virgatus* and *Leucaena leucocephala* have an average CP (17.8% vs. 25.2%) which is moderately rich in condensed tannins (8.3% DM) (Ramirez *et al.*, 2000; Ramirez *et al.*, 2001). Therefore, if it is used at appropriate levels, it can be fed safely and has the potentially useful to be used as a protein raw material to feed the ruminants without toxic symptoms. It has been reported that Leucaena should not be used as a major portion of the diet in non-ruminant animals because they are lower ability to tolerate mimosine than ruminants (Rushkin, 1984).

Alfalfa contains numerous secondary metabolites that are useful as human nutrition (Stochmal *et al.*, 2001). There are six phytonutrients in alfalfa including saponins (Oleszek, 1996; 2000), flavonoids (Herna´ndez *et al.*, 1991; Bisby *et al.*, 1994), condensed tannin (Cleef & Dubeux, 2019), coumestrol (Knuckles *et al.*, 1976), carotenoids, and tocols (Livingston, *et al.*, 1980; Hegsted & Linkswiler, 1980) (cited by Stochmal *et al.*, 2001). Due to the fact that the biological activities of flavonoids, it has recently been recognized as an active principal (antioxidant, cancer-preventing, and antimicrobial) (Packer *et al.*, 1999) which also have pharmalogical benefits (Mamta & Jyoti, 2012). Thus, these characteristics of the phytochemical composition of alfalfa offer potential sources for important health based compounds will improve our understanding of their nutritional value. Although parts of the alfalfa plant have most often been unspecified, a number of different flavonoids (Bisby *et al.*, 1994), for example, 47 varieties of alfalfa from USDA stocks, showed that all varieties had similar profiles to flavonoids (Stochmal *et al.*, 2001). Numerous research studies also suggest that a blend of antioxidants found in various phytochemical plants, vegetables and fruits of many types will provide better antioxidant quality when compared to antioxidants from a single food source. This research paper focuses on the nutritive value of alfalfa plants as a rich source of health benefits materials. We expected that they might be the potential of value-added raw materials sourced from alfalfa plants production and providing high-value food supplements in Thailand, or alternatively, as having a possible use as a feed ingredient in some ruminant diets.

Materials and Methods

Study area

The alfalfa *(Medicago sativa)* plantation was located at the experimental farm of the agricultural research facility of the Faculty of Agricultural Technology, Burapha University, Sa Kaeo Campus, Watthana Nakhon district, Sa Kaeo province in the eastern part of Thailand**.** The elevation was lower than 1,000 meters above sea level with dry and shallow sandy soil**.** The soil of the field crops in Sa Kaeo province was classified to be 49 series, including the Phon Phisai soil series ***(***Pp***)***, Sakon soil series ***(***Sk***)*** and Sa Kaeo soil series ***(***Ska***)***, and it is characterized by pH of 6**.**59 and low fertility**.**

Collection and chemical analysis of alfalfa plant

The alfalfa samples were randomly collected at 120 days after planting (Figure 1C) for chemical composition analysis of dry matter (DM), ash, crude protein (CP), ether extract (EE), crude fiber, tannin, total phenolic, total flavonoids and isoflavone. Shoot samples of about 20-30 cm from the tip of the alfalfa branches were taken. Also, samples of the alfalfa plants were cut and immediately chopped into 3-4 cm length with a mechanical forage chopper and dried at 60^{o}C for 48 h. Representative samples of alfalfa were later ground through a 1 mm screen and stored pending chemical analysis and being subjected to a proximate analysis. Chemical analyses of alfalfa samples were performed on each sample in three replications, and the analysis was repeated when the CV was > 0.05. Analytical DM was analyzed by drying samples at 135°C for 2 h, followed by hot weighing (AOAC, 1990; method 930.15). Ether extract was determined by using petroleum ether in a Soxtec System (procedure 948.15, AOAC, 1998). Crude fiber was determined with the standard methods of the AOAC (2010); method 962.09. The chemical analysis was expressed on the basis of the final DM. Determination of total tannins (as feed basis) was carried out by following the methods of Burns (1971) by submitting samples to the Feed analysis-2 Laboratory, Department of Animal Science, Faculty of Agriculture at Khamphaeng Saen, Kasetsart University, Khamphaeng Saen. Total phenolic content and total flavonoid compound were extracted and determined using the Folin-Ciocalteu method following the protocol of Kubola & Siriamornpun, 2011). Isoflavone extraction was determined by using the methods of Devi *et al*. (2009), and analyzed by high performance liquid chromatography (HPLC). Analysis was performed using Shimadzu LC-20AC pumps, a SPD-M20A diode array detector, and a LUNA C-18 column (4.6 x250 mm i.d., 5 µm). Analyses were made using three replications and the results were reported as averages with calculated standard deviations.

Morphological observation

Alfalfa plants were observed for morphological characterization on germination, seedling, stem and leaf shape, flowering period, inflorescence form and pod shape (Figure 1). All characteristics were compared to report of Undersander *et al*. (2011) that described the botany, vegetative stage and flowering stage of alfalfa. This observation showed that alfalfa characteristics were normally when it was grown at Sa Kaeo province.

Extraction and determination of total phenolics content

The extracts prepared from the freeze-dried alfalfa samples were approximately 5 g and were extracted with 20 ml of 80% methanol (V/V) on a shaking incubator set at 37°C for 12 h. After being filtered through a Whatman No.1 filter, the supernatant was mixed with other previously extracted supernatants of the sample under identical conditions and combined before being decanted into a vial and then stored at -20°C until the total phenolic content and the total flavonoid compounds were measured.

Determination of total phenolics content

The total phenolic content was determined using the Folin–Ciocalteu reagent as described by Kubola & Siriamornpun (2011) and gallic acid was used as standard. Three hundred microliters of alfalfa extract were mixed with 10% of the Folin–Ciocalteu reagent and 2 ml of 7.5% sodium carbonate (Na_2CO_3) solution. After 90 min incubation time at room temperature, the absorbance was measured at 725 nm using a spectrophotometer. The total phenolic content of the alfalfa extracts was calculated and expressed as gallic acid equivalents at a concentration of 1 to 1000 mg/L which was used as a standard. The quantitative results were expressed in grams of dry weight (mg GAE/gDW (sample)) based on the gallic acid standard curve.

Determination of total flavonoid compounds

Briefly, 500 µl of the alfalfa extract was mixed with 2.25 ml of distilled water following by the addition of 150 µl of 5% $NaNO_2$ solution in test tube. After 6 min, 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for another 5 min before 1.0 ml of 1 M NaOH was added. The mixture was mixed well using a vortex. The absorbance was measured immediately at 510 nm using a UV-vis spectrophotometer. The quantitative results were expressed in mg rutin equivalents in 1 g of dried sample (mg RE/g).

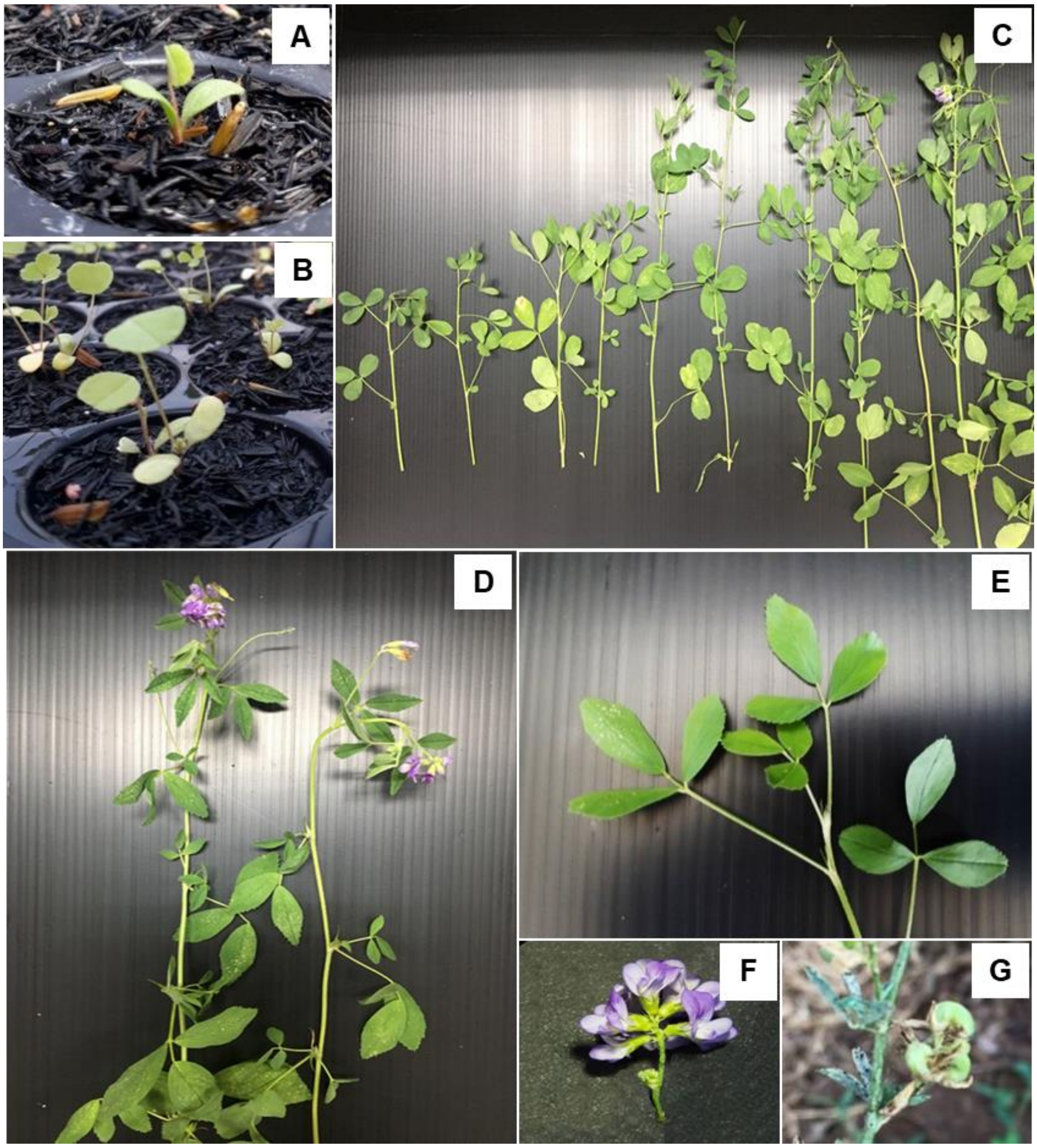

Figure 1: Characteristics of alfalfa plant: germination (A), seedling (B), samples collection at 120 days (C), flowering stage (D), compound leaf (E), inflorescence (F) and pod (G).

Results and Discussion

The results of a proximate analysis of the alfalfa plants showed that they contained DM about 91.44%. The CP was between 17.67 to 17.69%, ash 9.18 to 9.22%, crude fat 2.44 to 2.47% and CF 26.40 to 26.53% at the cutting interval of 120 days, respectively (Table 1). The CP was similar to the approximate value of alfalfa legume at cutting intervals of 30, 45, 60 and 75 days from between 18.1 down to 16.7%CP at Burapha University Sa Kaeo Campus, Watthana Nakhon District, Sa Kaeo province during the period from October 2018 to July 2019 [Study Topic: Effects of feeding Leguminosae on productive performance in goat meat]. (Unpublished raw data). A similar result was also recently published by Vintu *et al.* (2012), with values ranging from 24.3 (early buds), 21.3 (late buds) and down to 16.7%CP in full bloom in the harvest period of whole alfalfa plants with non-fertilizer. Although the forage value of alfalfa was high protein, there was low yield at 325.6, 619.1, 533.0, 597.3 kg/rai with cutting intervals of 30, 45, 60 and 75 days respectively, this demonstrates that it was alfalfa should be combined or mixed with other feedstuffs to make it suitable for the production of animal feed in Sa Kaeo province. It is therefore suggested that alfalfa should be harvested at the beginning of the bud formation period, so that the yield of CF will also decrease markedly. The decreases in %CF, or %NDF and %ADL content with decreasing cutting intervals can possibly be attributed to the cattle eating more because of its low fiber resulting in the feed flowing too rapidly through the intestine. This increases the digestibility and consume ability of the forage for ruminants which corresponds to Srisaikham & Lounglawan (2018) who reported that cutting a stand of sunnhemp (*Crotalaria juncea*), which was in the same Leguminaceae family, at 55 days of harvesting time would achieve a greater yield of CF than cutting at 30 days.

Table 1: Chemical composition of the branches of alfalfa (*Medicago sativa*) at harvesting intervals of 120 days.

Item	Alfalfa[1]	Alfalfa[2]	Alfalfa[3]	Alfalfa ($\bar{x}$)
DM (%)	91.46±0.03	91.44±0.03	91.43±0.05	91.44
Ash	9.22±0.12	9.18±0.15	9.19±0.14	9.20
CP (%)	17.68±0.10	17.69±0.08	17.67±0.12	17.68
EE (%)	2.44±0.01	2.46±0.02	2.47±0.02	2.46
CF (%)	26.50±0.11	26.40±0.13	26.53±0.10	26.48
Tannin (%)	2.75±0.07	2.81±0.13	2.79±0.10	2.78

[1] the representation of the first replicated of alfalfa results are an of the 3 values obtained for each set with a standard deviation, [2] and [3] the representation of the second and third replicated of alfalfa results are an average of the 3 values obtained for each set with a standard deviation respectively, DM = Dry matter, CP = Crude protein, EE = Ether extract

We investigated the composition of the phytochemicals (total phenolic content, total flavonoid compounds and isoflavone (daidzein and genisten) and the type and content of flavonoid compounds of all of the alfalfa. Details of the samples are described in Table 2 and 3. The results show that the alfalfa was detected in TPC (2.17 mg GAE/g), TFC compounds (2.02 mg RE/g), daidzen (46.72 µg/g) and genisten (15.38 µg/g) and flavonoid content including catechin (434.55 µg/g), rutin (46.67 µg/g), myricetin (1354.78 µg/g), luteolin (19.41 µg/g), quercetin (183.31 µg/g) and apigenin (2267.75 µg/g). Alfalfa is a known source of phytoestrogens that exhibits estrogenic activity in plants (Seguin *et al.*, 2004) and many groups of this substance can be identified as isoflavones and they also have high genistein, daidzein, and coumestrol (Romm *et al.*, 2010). Phytoestrogens can be found in 3 groups of plants, namely, legumes, cereals and grasses, all of which plants are used in the farming of ruminants. Hloucalová *et al.* (2016) reported that the phytoestrogen content (daidzein and genistein) in fresh cut of *Medicago sativa* L. harvesting at flowering stage was similar to the present study at 0.057 and 0.014 mg/g of DM respectively. Due to the current health-conscious consumer trends, there is a need for a variety of alternative foods in response to the demands of modern lifestyles and consumers have begun to choose foods or products which are beneficial for their health and which are derived from natural sources. Therefore, farmers who manage to feed cows with legumes (especially alfalfa or beans) should be able to produce high phytoestrogen milk which is useful as it composed of antioxidants. They will prevent the symptoms of premature ageing of humans. Phenolic and flavonoids compounds are both known as strong antioxidants which can also reduce the risk of cardiovascular disease (Rodrigues *et al.*, 2012). (Steinshamn *et al.*, 2008) showed that the supplementation of forage legumes increased the phytoestrogen in milk which has both health benefits for consumers as well as adding value to the milk produced by dairy goat farmers.

Table 2: Total phenolic content, total flavonoid compounds and isoflavone (daidzein and genisten) of alfalfa (*Medicago sativa*) at harvesting intervals of 120 days.

Sample	TPC (mg GAE/g sample)	TFC (mg RE/g sample)	Daidzein (µg/g sample)	Genisten (µg/g sample)
Alfalfa	2.17±0.02	2.03±0.01	46.72±0.01	15.36±0.01
	2.18±0.02	2.05±0.02	46.71±0.01	15.39±0.02
	2.15±0.02	2.01±0.01	46.74±0.02	15.37±0.01
Mean	2.17	2.02	46.72	15.38

TPC = Total phenolic content, (TPC was determined in comparison with standard gallic acid and the results expressed in terms of mg GAE/g), TFC = Total flavonoid compounds (TFC was determined in comparison with standard rutin and the results expressed in terms of mg RE/g), ($\bar{x}$)±SD) (n = 3).

Table 3: Type and content of flavonoid compounds of alfalfa (*Medicago sativa*) harvested at intervals of 120 days.

Sample	Flavonoid content (µg/g sample) (Mean±SD)						
	Catechin	**Rutin**	**Myricetin**	**Luteolin**	**Quercetin**	**Apigenin**	**Kaempferol**
Alfalfa[1]	432.76 ± 5.90	47.07 ± 3.62	1354.76 ± 82.94	19.46 ± 0.61	182.55 ± 10.23	2265.41 ± 90.32	nd
Alfalfa[2]	434.44 ± 5.93	46.49 ± 3.41	1356.81 ± 83.75	19.40 ± 0.58	184.67± 10.40	2270.33± 90.43	nd
Alfalfa[3]	436.45 ± 5.97	46.44 ± 3.44	1352.78 ± 82.39	19.36 ± 0.56	182.71 ± 10.36	2267.52 ± 90.37	nd
Total mean	434.55	46.67	1354.78	19.41	183.31	2267.75	-

Values were expressed as mean ± SD of triplicate measurements. 1 = the present of the first replicated of alfalfa results are an average of the 3 values obtained for each set with a standard deviation. 2 and 3 = the present of the second and third replicated alfalfa results are an average of the 3 values obtained for each set with a standard deviation respectively.

Conclusion

This study has shown that alfalfa can be a beneficial natural nutrient composition as contains relatively high in protein with the potential bioactive compounds. These characteristics suggest that alfalfa could be a promising source of nutritional value for health. However, research on the impacts of bioactive compounds from alfalfa legumes when it is used in the diets of dairy cattle or goats in Thailand is limited. Thus, it is recommended that the use of alfalfa legumes combined with the nutrients available from other roughage sources should be used for ruminants. These can be fed to ruminants to increased phytochemical activity in milk in order to benefit the health of consumers.

Acknowledgements

The author would like to thank Mr. Theeraphan Chumroenphat in the Laboratory Equipment Center, Mahasarakham University, Thailand, for the assistance.

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