Adama Science and Technology University

School of Applied Natural Science

Research report

On

Quantification of Bioactive Constituent D-Pinitol in Ethiopian Soybean

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July, 2017

Adama, Ethiopia

Abstract

Vegetable soybean (Glycine max) is well established legume in the human diet in all over the world. D-Pinitol is a natural product belonging to groups of Cyclitols. D-pinitol plays tremendous medicinal roles. The aim of this work is to quantify the bioactive constituent D-Pinitol from seeds, seed pods and leaves of soybean. D-Pinitol extraction of soybean was processed with methanolic extraction followed by quantification using a UV spectrophotometer in comparisons with the Caro pinitol®. The maximum wavlength and absorbance of the standard dissolved in laboratory reagent water were 193 nm and 0.904 respectively. The best-fit line, R^2, was found to be greater than 0.9983, indicating the D-pinitol's concentration has significant effect on D-pinitol's absorbance over a concentration range of 31.25 – 1000.0 µg/mL. The mean recovery of the method was 94.3 ± 8.06 %. The absorbance of the serially diluted standard of 31.25µg/nL, 62.5µg/mL, 125µg/mL, 250µg/mL, 500µg/L and 1000µg/mL at 193 nm is 0.032, 0.042, 0.096, 0.205, 0.375, and 0.714, respectively. The absorbance of the samples Nyala seed (so), Nyala seed (mz), Nova seed (so), Nova seed (mz), Nyala seed pod (so), Nova seed pod (so), Nyala leaf (so) and Nova leaf (so) at 193nm were 0.237, 0.267, 0.307, 0.204, 0.213, 0.276, 0.24 and 0.263, respectively. The quantity (gm) of D-pinitol within 25gm crude extract of Nyala seed (so), nyala seed (mz), nova seed (so), nova seed (mz), nyala seed pod (so), nova seed pod (so), nyala leaf (so) and nova leaf (so) were 16.2, 18.4, 21.3, 13.9, 14.5, 19, 16.5 and 18.1, respectively. The quantitative values of all varieties are closer to each other and this may be attributed to the similarity between the sampling areas, South Omo and Metekel zone. Generally, the Ethiopian soybean has high D-pinitol content. The pairwise comparison using the Student's t-test shows no significant difference, $p > 0.05$, between the content of D-pinitol in seed, seed pod and leaf.

Keywords: soybean, D-pinitol, UV spectrophotometer, quantification, t-test.

Acknowledgments

The authors would like to acknowledge Jinka Agricultural research center and Assosa agricultural research center for the provision of soybean samples. It is also the authors' deep pleasure to gratitude ASTU for provision of fund for the project.

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Acronyms

n.d.	no date
df	dilution factor
so	South Omo
mz	Metekel Zone

1. Introduction

A compound d-(+)-Pinitol (3-O-methyl D-Chiro inositol) is a natural product belonging to groups of Cyclitols (cyclic polyol) (Chaubal et. al., 2005).

D-pinitol has been appears to act downstream in the insulin-signaling pathway to mimic the effects of insulin i.e. antidiabetic activities (Davis et. al., 2000; Fonteles et. al., 1996; Misra and Siddiqi, 2004; Al-Yusuf, 2007; Van Wyk, 2008). Thus, in humans, this compound may assist with diabetes by lowering blood sugar levels and making the glucose more available to the cells i.e. glucose metabolism (Bates et. al., 2000; Davis et. al., 2000; Narayanan et. al., 1987; Numata et. al.,1979) and promoting the glycogen synthesis (Davis et al., 2000). Pinitol can also drive creatine and other nutrients into muscle cells (Davis et. al., 2000; Van Wyk and Albrecht, 2008). Furthermore, an insecticidal effect has been described caused by this cyclitol on the larval growth of *Heliotis zea, Aedes egypti and Culex quinquefasciatus* (Chaubal et. al., 2005; Dreyer et. al., 2005).

It was also found to be responsible for activities of chronic complication obesity; Hyperlipidemia; Dyslipidemia atherosclerosis; Hypertension; malnutrition, stress, aging, Hyperuricemia & Anthelmintic (Chaubal et. al., 2005) and more recently, for the ability to modulate the immune response by interacting with dendritic cells (DCs) maturation (Lee et. al., 2007).

It also exerts multifunctional properties, including anti-inflammatory activity (Al-Yusuf, 2007; Priyanka et. al., 2014; Singh et. al., 2001), preventing wasting and inflammation in HIV/AIDS patients (Van Wyk and Albrecht, 2008). It prevents cardiovascular diseases (Chaubal et. al., 2005; Gupta et. al., 2013; Kim et. al., 2005) and used as an antioxidant (Gupta et. al., 2013). Moreover, D-pinitol is an active principle feeding stimulant activities (Al-Yusuf, 2007).

Animal studies showed that D-Chiro-inositol is synthesized endogenously in small quantities, while in human most D-Chiro-inositol is obtained from dietary pinitol. (Priyanka et. al., 2014).

In plants, this bioactive molecule is an important stress metabolite and its accumulation may be related to plant tolerance to water deficit stress (Guo and Oosterhuis, 1997). It occurs in species adapted to temperature extremes, such as jojoba (*Simmondsia chinensis*) (Dittrich and Korak, 1984) and maritime pine (*Pinus pinaster*) (Nguyen and Lamant, 1988). It has been isolated from various plants (Davis et. al., 2000; Narayanan et. al., 1987; Numata et. al., 1979), trees and citrus fruits (Davis et. al., 2000). It's found primarily in legumes (Davis et. al., 2000) and is a major component of the soybean plant (Ford, 1984; Guo and Oosterhuis, 1993; Phillips et. al., 1982). In soybeans, pinitoI has been found in various tissues, including leaf blades, petioles, stems, roots, nodules and seeds (Horbowicz and Obendorf, 1994; Kawai and Kumazawa, 1982).

Many pharmaceutical preparations of D-pinitol are marketing the popular D-pinitol products under the trade name Biochem GlucoLean® and Inzitol® can help to facilitate glycogen or circulating sugar into metabolically active tissues (Al-Yusuf, 2007). Thus, world is now moving towards the natural product derived drugs like D-pinitol that tend to cure diseases without any toxic side effects. Despite of these, to the best of the authors' knowledge, D-pinitol was yet not investigated from soy bean from Ethiopia. Therefore, the aim of the current study is to extract and quantify D-pinitol, from soybean leaves, seeds, and seed pods.

2. Literature Review

The origin of soybean (*Glycine max* (L.) Merrill) cultivation is China. With its countless and varied uses, soybean, is an important crop at the global level. This crop has the highest protein content and the highest gross output of vegetable oil among the cultivated crops in the world. In 2007, the total cultivated area of soybean in the world was 90.19 million ha and the total production was 220.5 million t The USA, Brazil, Argentina, China and India are the major soybean producing countries (FAO, 2009).

Vegetable soybean is well established legume in the human diet in all over the world. The positive health benefits of soy have greatly increased consumer awareness of soy products and created a market potential for soy products. D-pinitol is the main compound of the soybean found as large quantities in soy foods and it occurs in about 1% of the dry weight of soybean meal (Davis et. al., 2000; Phillips et. a., 1882). Naturally occurring isomers of inositol are *myo*inositol, C*hiro*-inositol, *scyllo*-inositol, *muco*-inositol, and neo-inositol with *Myo*-inositol being the most abundant (Wang et. al., 1990).

Soybean is introduced to Ethiopia where it has been grown experimentally at a number of medium altitude locations in Assella, Shewa and on some state farms, particularly in Gojam (Thulin, 1989). Soybean, one of the Leguminosae families is the main sources of D-pinitol and inositol. An outstanding characteristic of soybean plant is its ability to produce large amounts of the carbohydrate pinitol. Pinitol and the closely related inositols are currently undergoing widespread investigation for their various biological activities and nutritional value (Barar, 2000). Pinitol is an anti-diabetic biomarker and has pharmacological significance which is highly remarkable and there is a volley of reports on its use in medicinal formulations (Singh et. al., 2011). Pinitol has immense pharmacological properties as it is best owed with anti-diabetic, anti-inflammatory, antioxidant, and immunosuppressive potential and is used in the treatment of hypertension, rheumatism, cardiovascular diseases, AIDS, and neurological disorders (Kim et. al., 2005).

The compounds pinitol (Figure 1) is belonging to groups of Cyclitols (cyclic polyol). Pinitol (3-O-methyl D-chiro inositol) (Chaubal et. al., 2005; Van Wyk and Albrecht, 2008) is a natural product of cyclitol group occurring mainly in its (+) form in certain leguminous plants, soya foods and was found to be responsible for hypoglycaemic activities, antidiabetic (Misra and Siddiqi, 2004).

Diabetes mellitus is a chronic disease that requires long-term medical attention. Since ancient times, plants have been an exemplary source of medicine for Diabetes. A wide array of plant derived active chemical compounds has demonstrated active constitute with their possible use in the treatment of Diabetes Mellitus (Marles and Farnsworth, 1995). Alkaloids, glycosides, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, and amino acids are primary and secondary metabolites derived from soybean extract. Antidiabetic effects can be studied *in vivo* using animal models or *in vitro* using a variety test systems. In vitro tests can play a very important role in the evaluation of the antidiabetic activity of drugs as initial screening tools where the screening of large number of potential therapeutic candidates may be necessary (Yadav et. al., 2008). Thus, this study provides the information of various *in vitro* studies used in antidiabetic assessment, which can establish mechanisms for the antidiabetic activity of the drug. Synthetic drugs are likely to give serious side effects in addition to they are not suitable for intake during conditions like pregnancy (Larner, 1985).

The pinitol constituents of soy plant were expected to vary with the variety and geographical location. The present work was carried out for the quantification of bioactive constituents (D-pinitol) from the extract of Ethiopian soybean leaves, seeds, and seed pods. To the best of the authors' knowledge, there were no studies on quantification of bioactive constituent D-pinitol from Ethiopian soybean.

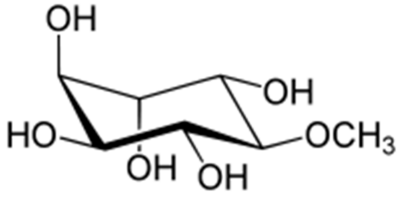

Figure 1. Chemical structure of pinitol

3. Methodology

3.1 sample collection

Seed, seed pod and leaf samples were collected from the research fields of Jinka agricultural research center and Assosa agricultural research center from the research fields during harvest season.

3.2 Extraction Method

The experiment was conducted at applied chemistry laboratory, ASTU, from January 2017 to April 2017.

Seed, seed pod and leaf was ground. Powdered material was kept for soaking in petroleum ether for 24 hours and filtered. The process was repeated three times for residue. Similar soaking was repeated by methanol (Table 1, appendix 1). All filtered portions were combined and evaporated in a rotavapour to give the brown mass (seed and seed pod extracts) and greenish (leaf extract) at 45-50^{0}C to yield a crude extract residue. The extract keeps in refrigerator for further investigation.

3.3 D-pinitol standard optimization and sample dilution

The Caro pinitol® (pinitol from carob), Amicogen, Inc., was used as standard. The standard was dissolved in laboratory reagent water for UV spectrometer measurement and the maximum wavelength of the absorbance was recorded (193 nm). Then the standard was prepared with the concentration of 31.25µg/ml, 62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml to check the absorbance linearity at 193 nm. The absorbance of the serially diluted standard of 31.25µg/ml, 62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml at 193 nm were 0.032, 0.042, 0.096, 0.205, 0.375, 0.714 respectively increases linearly.

The 25 mg of methanol extract was dissolved in 25 ml detergent water for UV spectrometer measurement as a stock solution of each sample. Then 100 µl of the stock solution was mixed with 5ml of detergent water (i.e. DF = 5ml H_2O + 0.1ml sample/0.1ml = 51).

3.4 D-pinitol quantification

Eight samples Nyala seed from south omo, Nyala seed from Metekel zone, Nova seed from south omo, Nova seed from Metekel zone, Nyala seed pod from south omo, Nova seed pod from south omo, Nyala leaf from south omo, Nova leaf from south omo were quantified and compared with the standard as references.

4. Results and Discussions

4.1 D-pinitol standard optimization

The sterile, deionized water was used as solvent as using methanol solvent could not give the clear peak. The lowest wavelength of measurement with the water solvent is 190nm (Koplik, n.d.). The measurement of standards and the samples attained this criterion.

The D-pinitol standard curve was determined using 31.5 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000µg/ml by serial dilution from a 1 mg/ml pinitol standard. The best-fit line, R^2, was found to be greater than 0.9983, indicating the D-pinitol's concentration has significant effect on D-pinitol's absorbance over a concentration range of 31.25 – 1000.0 µg/ml (figure 2). i.e. ~99.8% of the variance in the data is explained by the line and 0.02% of the variance is due to unexplained effects. The mean recovery of the method was 94.3 ± 8.06 %. Therefore, the followed method for D-pinitol quantification is precise and accurate.

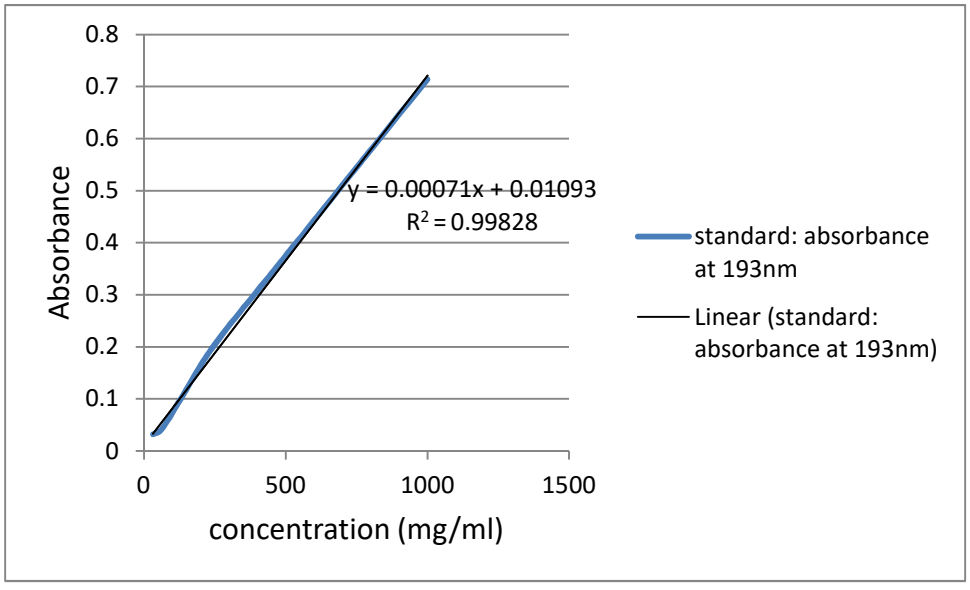

Figure 2. D-pinitol standard absorbance at 193nm (standard/calibration curve)

4.2 D-pinitol standard absorption and wavelength (nm)

The maximum absorption of the D-pinitol standard at 193 nm is 0.904 (figure 3). This is the proximate figure with Mininath et. al., 2015 reported as 229nm. However, the quoted author used methanol solvent whose lowest wavelength of measurement is 205nm (Koplik, n.d.).

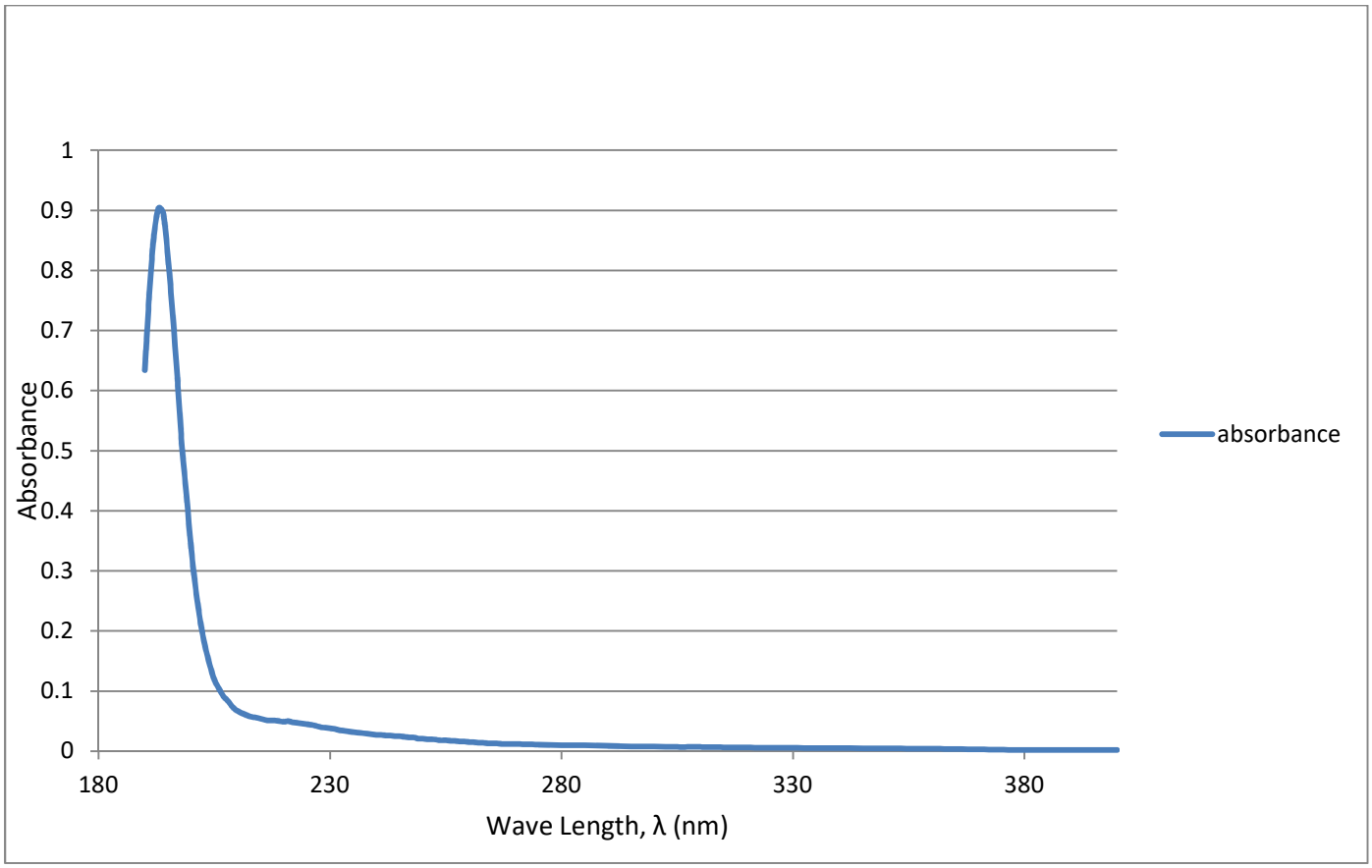

Figure 3. Max absorbance of the D-pinitol standard at 193nm

4.3 Samples' absorption and wavelength (nm)

The absorption of a selected representative sample (Nova seed from Metekel zone) is 0.192 at 193 nm which is proximate to the result of the standard, 0.904.

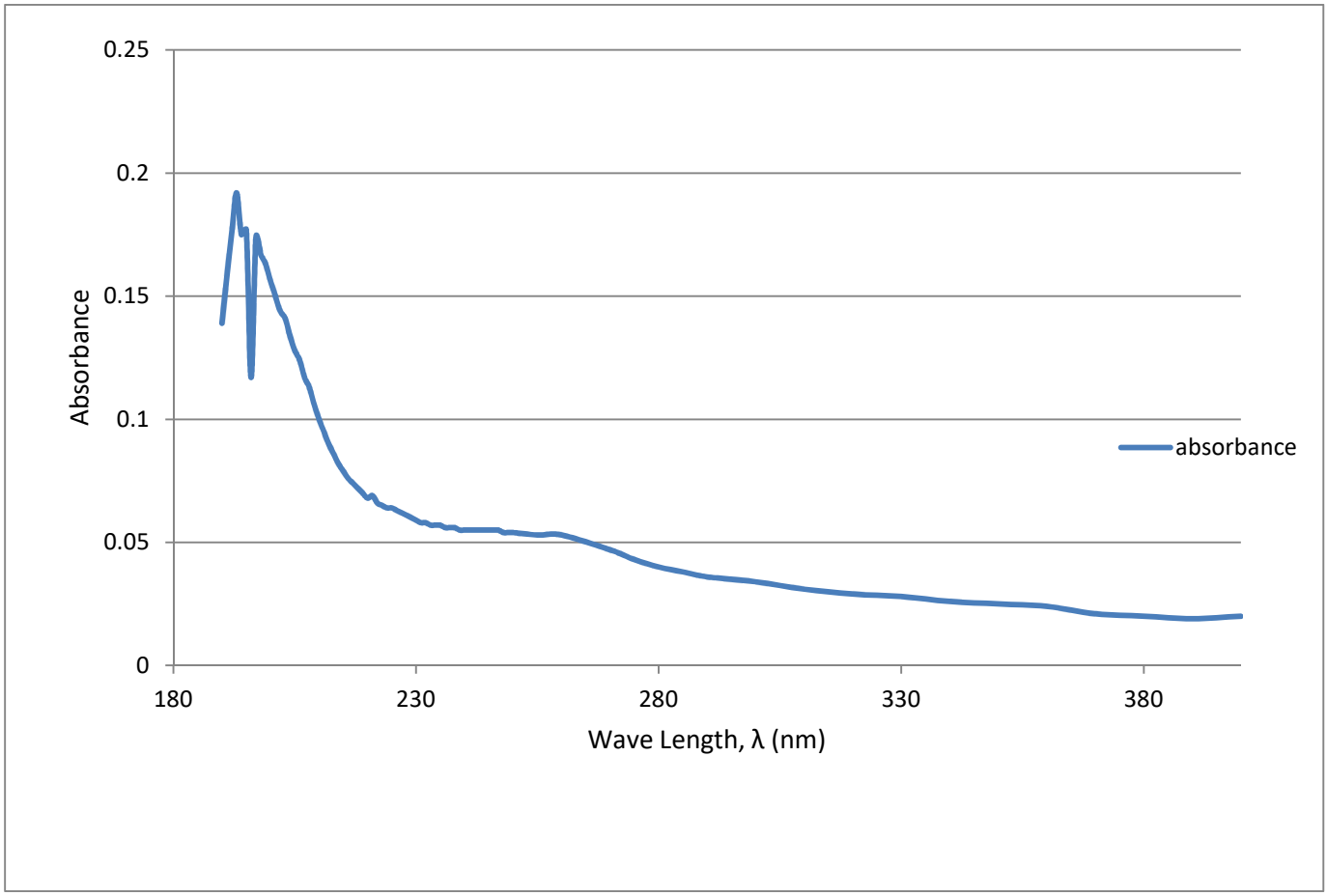

Figure 4. absorbance of randomly selected representative seed sample (Nova-mz)

4.4 The concentration and the absorbance of samples

The absorbance of the samples Nyala seed (so), Nyala seed (mz), Nova seed (so), Nova seed (mz), Nyala seed pod (so), Nova seed pod (so), Nyala leaf (so) and Nova leaf (so) at 193nm respectively were 0.237, 0.267, 0.307, 0.204, 0.213, 0.276, 0.24 and 0.263. The

concentration of d-pinitol in each sample are calculated using the formula from the trendline equation of y = 0.00071x + 0.01093. Accordingly, The quantity (gm) of d-pinitol within 25gm crude extract of Nyala seed (so), Nyala seed (mz), Nova seed (so), Nova seed (mz), Nyala seed pod (so), Nova seed pod (so), Nyala leaf (so) and Nova leaf (so) is 16.2, 18.4, 21.3, 13.9, 14.5, 19, 16.5 and 18.1 respectively. This concentration range is in agreement with the concentration range of the standard. Therefore, the isolated compound from tissues of soybean leaf, seed and seed pod is D-pinitol. Even though, the variation of concentration of samples from different areas is expected as the accumulation of pinitol is associated with heat stress, drought stress and salt stress (Ford 1984; Guo and Oosterhuis, 1997; Keller and Ludlow, 1993; Manchanda and Garg, 2008; Nguyen & Lamant 1988; Streeter, et. al., 2001), the quantitative values of all varieties are almost proximate to each other. This may be attributed to the similarity between the sampling areas, South Omo and Metekel zone.

D-pinitol found in all tissues of soybean, leaf, seed and seed pod. This is in agreement with previous reports, D-pinitol is present in soybean leaves (Dittrich & Brandl, 1987; Streeter et. al., 2001); leaves are the major site of pinitol synthesis (Dittrich and Korak, 1984; Paul and Cockburn, 1989; Ruis and Hoffmann Ostenhof, 1969), and pinitol has been suggested as the principal transport carbohydrate in soybean petiole exudates (Guo and Oosterhuis, 1993; Kawai et. al., 1985). Soybean plants exposed to high temperature had significantly higher pinitol contents in leaves and stems, and the pinitol content in stems was almost doubled. Pinitol content in soybean leaves, stems and roots are generally in a descending order: pinitol is transported out of the leaves to stems and roots (Guo and Oosterhuis, 1993; Kawai et. al., 1985). Free D-pinitol concentration is highest in seed coats and lower in the axis and cotyledons of soybean seeds (Kuo et. al., 1997), suggesting the transport of D-pinitol from leaves to seeds (Ralph et. al., 2004). The presence of pinitol in tissues other than the seed is important in the matter of using seed for other purposes and getting other tissues is simpler than seed.

Statistical analysis between two samples was performed using the Student's t-test (Lin et. al., 2013). The pairwise comparison was done in seed, seed pod and leaf samples

collected from south omo. In all cases, $p > 0.05$. i.e. no significant difference between the d-pinitol content of seed, seed pod and leaf.

As the sample collected from metekel zone is only a seed, pairwise comparison was not made. Further study is needed to analyze the pinitol content of different tissues of Ethiopian soybean.

Even though, it needs to investigate the relative constituent of D-pinitol with other phytochemicals, the D-pinitol content of the Ethiopian soybean is high as compared to different previous reports conducted other than Ethiopia. For example, Davis et. al., 2000 reported as Pinitol constitutes 1% of the dry weight of soy meal. Singh et. al., 2001; Geethan and Prince, 2008 reported that mature and dried soybean seeds contain up to 1% D-pinitol.

5. Conclusions and Recommendations

In the present investigation on the basis of UV spectrophotometer data, it was concluded that the isolated compound from the methanolic extract of Glicine max is a D-Pinitol. The D-pinitol content of the Ethiopian soybean is high compared to previous reports.

It is the authors' recommendation that different methods are better to be used for further identification and characterization of the D-pinitol from the Ethiopian soybean. More Investigations are needed to address the relationship between the pinitol concentration level and different environments in Ethiopia. It is also very useful if the relative constituent of D-pinitol compared with other phytochemicals found in Ethiopian soybean.

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7. Appendices

Appendix 1: Table 1. pinitol extraction and quantification

No.	Sample type	Amount of powder used for soaking (gm)	Gram obtained		Pinitol (gm) in 25gm crud extract	Absorbance at 193nm
			After PEt (500 mL)	After MeOH (500 mL) (solid)		
1	Nyala Seed (So)	100	10.8 (oil→liquid)	5.5	16.2	0.237
2	Nyala Seed (Mz)	100	14.7 (oil→liquid)	7.5	18.4	0.267
3	Nova Seed (So)	100	14.5 (Oil→liquid)	5.5	21.3	0.307
4	Nova seed (Mz)	100	12 (oil→liquid)	5.9	13.9	0.204
5	Nyala seed pod (So)	100	1.8 (solid)	2.3	14.5	0.213
6	Nova seed pod (So)	100	1 (solid)	2	19	0.276
7	Nyala leaf (So)	100	1.4 (solid)	14.7	16.5	0.24
8	Nova leaf (So)	100	2.6 (solid)	16.5	18.1	0.263