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Phytochemical, proximate composition, amino acid profile and characterization of Marijuana (*Cannabis sativa* L.)

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Abstract

The phytochemical and proximate compositions, amino acid profile and characterization of *Cannabis sativa* leaves, stem and seeds were conducted to provide baseline information on its potent in feed materials for its subsequent utilization in supplementing fish nutrition in aquaculture. Phytochemical analysis of the leaves revealed the presence of alkaloids, flavonoids, cardiac glycosides, resins, terpins and steroids while the proximate composition had elevated levels of 6.87% moisture, 23% crude protein, 19.97% lipid and 11.8% Ash; 18.95% fibre and 39.70% NFE in the stem and 25.36% crude fiber content in seeds. *C. sativa* leaf contains 9 Essential Amino Acids (EAA), which have good concentration of methionine and lysine. Characterization of the leaf extracts revealed six clearly-pigmented spots with the highest travelled as cannabinol (CBN). The implications of these findings indicate that *C. sativa* has potential inclusion in fish feeds requirement, ameliorating stress conditions during handling, sampling and transportation as well as possible utilization to anesthetize fish going by the array of the bioactive compounds present in the crude leaf extracts of the plant.

Keywords: Phytochemicals, Proximate composition, Amino acid, *Cannabis sativa*.

Introduction

Cannabis is a cosmopolitan annual weedy plant that is grown in many parts of the world. It requires little fertilizer, resist pests, crowds out weeds, relatively easy to grow, does well as an organic crop, grows quickly requiring 70 to 110 days to maturity and hence an abundant supplier of its extremely valuable raw materials.[1] D'Mello, reported that the different varieties of cannabis can have different levels of medicinal and nutritional values.[2] The dry fruiting and flowering tops and leaves of the female cannabis plant contain significant quantities of the psychoactive constituents such as tetrahydrocannabinol and thus known as the drug containing parts with its strongest form being the pure resin.[3] Studies have shown that Cannabis has been used as a pest repellent and pesticide in a variety of formulations. It has been planted as a companion crop to deter insects, nematodes, fungi, and weedy plants. Dried leaves and flowers have repelled or killed insects, mites, nematodes, and weeds.[4] Though tetrahydrocannabinol (THC) is considered the primary active component of the cannabis plant, various scientific studies have suggested that certain other compounds like cannabidiol (CBD) may also play a significant role in its psychoactive effect.[5, 6] At least sixty six (66) other cannabinoids are also present in cannabis including cannabinol (CBN) cannabidiol (CBD) and tetracannabivarin (THCV), which can result in different

effect from those of THC alone.[7] Cannabidiol (CBD) a major component of cannabis possesses sedative properties[8] and clinical trial showed that it reduces the anxiety and other unpleasant psychological side effect provoked by THC.[9] CBD actually kills bacteria with greater potency than THC and thus, cannabis may have less microbial contamination than other medicinal plants (herbs); an important consideration for immuno-compromised individuals.[10] The active ingredients in *C. sativa* parts (leaves, seeds, fruits and bark) have varying potencies and mode of action depending on whether applied directly or in form of either aqueous or alcohol extracts.[11] *C. sativa* has analgesic, anti-emetic, anti-inflammatory, sedative, anticonvulsive, and laxative actions and clinical studies have demonstrated its effectiveness in relieving nausea and vomiting following chemotherapy treatments for cancer.[12] The whole hemp plant (including stalk and leaves) would be, due to its high fibre content, a suitable feed material for ruminants (and horses), and daily amounts of 0.5 to 1.5 kg whole hemp plant dry matter (DM) could likely be incorporated in the daily ration of dairy cows.[13] The aim of this study was to investigate the presence of bioactive compounds in the crude leaf extracts of *C. sativa* which may provide baseline information for its subsequent utilization in supplementing fish nutritional requirements, anaesthesia stress management, as an alternative natural fish biotic as well as its overall importance in sustaining biodiversity.

Materials and Methods

Procurement of Cannabis

A total weight of 500 g of Marijuana (*Cannabis sativa* L.) was obtained from the National Drug Law Enforcement Agency (NDLEA), Jos Plateau State Nigeria, command strictly for scientific research. Visual examination revealed that the material consisted of long leaves, stems/twigs and seeds along with some powdery leaves

The leaves (405 g) were carefully sorted out from the stem/twigs (73.75 g) and seeds (17.21 g) by handpicking and then separately powdered using mortar and pestle and sieved through a metal sieve (90 µm mesh size). The powdered *C. sativa* leaves (398.86 g), stem (65.33 g) and seeds (14.62 g) were separately stored in airtight polyethylene bags.

Phytochemical Analysis

The phytochemical analysis was for the determination of the alkaloid, flavonoids, tannins, phenols, cardiac glucosides, terpenes and steroids, resins volatile oils, and balsam present in crude leaf extract of *C. sativa*. A stock solution of the crude leaf extracts of *C. sativa* was prepared for the phytochemical analysis by macerating 5g of the dried powdered leaves in 200 ml of petroleum ether for 24 hours at 25ºC. This resulted to 300 mg weight of oily resin after evaporating the solvent at room temperature (26 ± 1ºC). To this weight (300 mg), 25 ml of acetone was used to dissolve the resin resulting to a solution 12 mg/ml. From this concentration (12 mg/ml), quantities were drawn for phytochemical analysis as described by the methods of Soforowa *et al*. and Wagner, *et al*.[14, 15]

Alkaloids were determined by adding few drops of Dangendroff"s reagents in 2 ml of petroleum ether crude leaf extract of cannabis in a test tube. The resultant mixture was observed for colour change usually orange to deep orange coloration.

Saponins were determined by adding 10ml of distilled water to 2 ml of the petroleum ether crude leaf extract of cannabis in a test tube. It was shaken vigorously for 1 minute and allowed to stand for 30 seconds after which 3 drops of Olive Oil was added and observed for color change usually dark brown coloration.

Tannins-(Ferric Chloride test) was determined by adding three drops of 10% ferric chloride ($FeCl_2$) in 2 ml petroleum ether crude leaf extract of Cannabis which was diluted with 4 ml of distilled water. The resultant mixture was observed for color change usually reddish precipitates formed

Flavonoids (Lead acetate test) was tested for by adding 2 ml of 10% lead acetate solution in 2 ml of the petroleum ether crude leaf extract of Cannabis in a test tube plus 2 ml of 10% lead acetate solution. This was allowed to stand for 10 seconds after which the mixture was observed for color change usually yellowish coloration and precipitate formed.

Cardiac Glucosides (Keller-Killiani test) was determined by adding 2 ml of glacial acetic acid and 1 drop of ferric chloride ($FeCl_2$) to 1 ml of the petroleum ether crude leaf extract of cannabis in a test tube. Finally, 1 ml of concentrated tetraoxosulphate (V1) acid (H_2SO_4) was carefully introduced in a slanting position down the side of

the test tube and was observed for brown ring inter phase formation

Terpenes and Steroids (Burchard test) were tested for by adding 1 ml of anhydrous acetic acid to 2 ml of the petroleum ether crude leaf extract of cannabis in a test tube. Concentrated tetraoxosulphate (V1) acid (H_2SO_4) was carefully added down the side of the test tube and was observed for a reddish color change and inters phase formation

Balsams were determined by adding 2 ml of alcohol ferric chloride to 2 ml of the petroleum ether crude leaf extract of cannabis in a test tube. The mixture was then warmed over a Bunsen flame for 5 seconds and was observed for reddish brown color change

Volatile Oils were determined by adding 0.1ml of dilute sodium hydroxide (NaOH) followed by 0.5ml of hydrochloric acid (HCL) to 2 ml of the petroleum ether crude leaf extract of cannabis in a test tube. This was then allowed to stand for 5 seconds and observed for a light blue color change and precipitate formed

Resins were determined by adding 2 ml of acetic anhydride to 2 ml of the petroleum ether crude leaf extract of cannabis in a test tube. Three drops of concentrated tetraoxosulphate (V1) acid, (H_2SO_4) was carefully added and then observed for a violet color change.

Proximate Analysis

The proximate composition of *C. sativa* leaves was determined using the standard methods of the Association of Official Analytical Chemists.[16] The parameters examined include, moisture content, crude protein, crude fat, crude fiber and ash content. The mean values from duplicate replicates were converted to percentage dry weight.

Amino Acid Analysis

The amino acid profile of the leaves, stem and seeds were done following the methods described by Spakeman *et al*. using Technicon TSM 1 Model DNA 0209 amino acid analyser.[17] Weights of 0.5g of the finely (90 µm) powered cannabis leaves were poured into a 250 ml Erlenmeyer flask. 50 ml of chloroform was added to the flask. 10 drops of glacial acetic acid was added to the mixture and allowed to stand for 30 minutes at room temperature (25ºC) and filtered through a funnel choked with non-adsorbent cotton wool. The filtrate was evaporated at 45ºC to dryness and the resulting residue was dissolved in 1ml chloroform. The mixture was then used for spotting on the developed reactivated Thin Layer Chromatography (TLC) plates.[18]

Characterization of Leaf Pigment

The procedure for the characterization of active ingredients of the leaves involves transparent glass plates (20x20 cm) which were rinsed with distilled water and allowed to dry in hot air oven at 40ºC for 30 minutes. The plates were removed and allowed to cool, then coated with a 0.25 mm thick layer of silica gel (60F254 Merck, Darmstadt) slurry (30 g gel + 65 ml distilled water), dried at room temperature and later reactivated at 110ºC for 40 minutes before use following the methods described by UNODC.[18] The solvents used for the development of the TLC Chamber were a mixture of heptane/ dichloromethane / butan-2-one; 83:5:12 volume ratio. The developing solvent mixture was placed into the TLC chamber and completely covered the bottom of the chamber to a depth of 0.5 cm. The chamber was kept closed and shaken gently to allow for solvent vaporization and kept standing for 1 hour to enable adequate saturation of the chamber with the developing solvent.[19] The reactivated TLC plates (20x20 cm) were carefully marked with pencil line 2 cm from the bottom. Using a Pasteur's micropipette, 1 drop of the petroleum ether crude leaf extract was applied onto the TLC plate and allowed to dry thoroughly before loading into the TLC chamber.[19] The loaded TLC plates were carefully introduced into the saturated TLC chamber with the sample line towards the bottom. The plates were allowed to sit on the bottom of the chamber with the extract line just above the extraction solvent and leaned against the wall of the chamber. The TLC chamber was covered and allowed to stand undisturbed for 1 hour. When the solvent front had reached 12cm, the plates were removed and air-dried at room temperature (25ºC).[15] A few drops of iodine crystals were poured into an empty-dried TLC chamber and the chamber lid covered to allow the iodine to sublimate for 20 minutes after which the chamber was opened and the pigment extracted TLC plates were reloaded into the chamber, lidded and allowed to stand for 2 hours for color intensification.[15]

Results

Test results of the phytochemical screening of *C. sativa* leaf showed that the Dragendoff's reagent test produced orange coloration confirming the presence of alkaloids.

There was no visible change in the appearance of the leaf extracts after conducting tests for the presence of saponins, tannins, balsam and volatile oils implying that these phytochemicals are not present in the leaves of *C. sativa*. The presence of light yellow, reddish brown, violet, reddish brown color at the interphase between acetic acid anhydride layer and sulphuric acid layer confirmed the presence of flavonoids, cardiac glucosides, resins, terpenes and steroids respectively in the leaf extract of *C. sativa* (Table 1).

Table 1: Qualitative phytochemical screening of crude leaf extract of *Cannabis sativa* obtained from Jos, Plateau State, Nigeria

PHYTOCHEMICAL	QUALITY	COLOR	TEST
Alkaloid	+ + +	Orange	Dragendoff's
Saponin	-		
Flavonoids	+ +	Light Yellow	Lead acetate
Tannins	-		
Cardiac Glycosides	+ + +	Reddish Brown	Keller-Killani
Balsam	-		
Phenols	-		
Terpenes & Steroids	+ + +	Reddish Brown	Burchard
Resins	+ + +	Violet	
Volatile Oils	-		

+++High presence ++ moderate presence - Absence

Test results of the proximate compositions of the leaves, stem and seeds are presented in Table 2. The leaf revealed 6.87% moisture with lipid content of 19.97%. The crude protein content gave a value of 23.78% protein, while the crude fibre was 18.95%. Ash content recorded a value of 11.18%; the stem had 17.20% crude protein, 23.14% crude fibre, ether extract of 19.97%, ash 11.18% and moisture content of 6.87%. The stem proximate contents showed crude protein content of 17.20%, crude fibre 23.14%, ether extract 8.02%, ash 6.78% while the moisture content recorded 5.16%. Test results of the seeds indicated crude protein content of 20.19%, crude fibre 25.36%, ether extract of 9.31%, ash 7.20% and moisture value of 5.91% with nitrogen free extract (NFE) values of 19.25, 39.70 and 32.03% for the leaves, stem and seeds respectively, obtained by finding the difference between the summed proximate values from 100 percentage (Table 2).

Table 2: Proximate Composition of the Leaves, Stem and Seeds of *Cannabis sativa* obtained from Jos, Nigeria

Proximate Composition (%)	Leaf	Stem	Seeds
Crude Protein	23.78	17.20	20.19
Crude Fibre	18.95	23.13	25.36
Ether Extract	19.97	8.02	9.31
Ash	11.18	6.78	7.20
Moisture	6.87	5.16	5.91
NFE	19.25	39.70	32.03

Test results of the amino acid (AA) profile of *C. sativa* leaf stem and seeds revealed that the leaves contain concentrations (g/100g protein) of Lysine (3.84), Histidine (2.21), Arginine (4.32), Aspartic acid (8.25), Threonine (2.26), Serine (3.15), Glutamic acid (10.0), Proline (2.85), Glycine (2.79), Alanine (4.03), Cystine (0.79), Valine (3.91), Methionine (0.89), Iso leucine (3.23), Leucine (7.10), Tyrosine (3.02)and Phenylalanine (3.94). The stem revealed a comparative linear decrease in the amino acids concentrations (g/100g protein) of Lysine (0.86), Histidine

(1.01), Arginine (2.59), Aspartic acid (2.11), Threonine (1.99), Serine (0.49), Glutamic acid (4.24), Proline (0.61), Glycine (1.10), Alanine (1.06), Cystine (0.52), Valine (0.75), Methionine (0.55), Iso leucine (1.01), Leucine (3.00), Tyrosine (0.95) and Phenylalanine (2.06) from those obtained in the leaves. The same trend was found with the amino acids concentrations (g/100g protein) of the seeds as follows: Lysine (1.24), Histidine (0.69), Arginine (3.11), Aspartic acid (1.55), Threonine (1.71), Serine (0.33), Glutamic acid (7.73), Proline (0.51), Glycine (0.38), Alanine (3.49), Cystine (0.31), Valine (1.10), Methionine (0.31), Iso leucine (0.57), Leucine (2.13), Tyrosine (1.27) and Phenylalanine (0.86). This is however not exactly true when the stem and seeds are compared where higher amino acids concentrations were found in eleven amino acids from the stem and only six were recorded from the seeds as being higher than those obtained from the stem (Table 3).

Table 3: Amino acid composition of the leaf, stem and seeds of *Cannabis sativa* obtained from Jos, Plateau State, Nigeria

Amino acid	**Concentration (g/100g protein)**			
Profile	**LEAF**	**STEM**	**SEEDS**	***Houlihan *et al.***
Lysine	3.84	0.86	1.24	NG
Histidine	2.21	1.01	0.69	2.22 *
Arginine	4.32	2.59	3.11	5.82 *
Aspartic acid	8.25	2.11	1.55	NG
Threonine	2.26	1.99	1.71	4.55 *
Serine	3.15	0.49	0.33	NG
Glutamic acid	10.00	4.24	7.73	NG
Proline	2.85	0.61	0.51	NG
Glycine	2.79	1.10	0.38	NG
Alanine	4.03	1.06	3.49	NG
Cystine	0.79	0.52	0.40	NG
Valine	3.91	0.75	1.10	5.65 *
Methionine	0.89	0.55	0.31	3.54 *
Isoleucine	3.23	1.01	0.57	4.05 *
Leucine	7.10	3.00	2.13	7.35 *
Norleucine	NA	NA	NA	NG
Tyrosine	3.02	0.95	1.27	7.80 *
Phenlyalanine	3.94	2.06	0.86	7.80 *

NA= Not determined; NG= Not given

The results of the thin-layer chromatography revealed six (6) clearly pigmented spots which were compared with the earlier findings of Perkin-Elmer Corp.[20] Wagner *et al.*[15] and the United Nations Division on Narcotic Drugs (UNDND).[21] The thin layer chromatography column obtained in this investigation is presented as Plate 1. From

plate 1, the highest travelled substance is cannabinol (CBN). This was closely followed by tetrahydrocannabinol (THC). The next travelled substance was cannabidiol (CBD) which showed a higher mobility range than cannabidioc acid (CBDA) which in turn performed better than tetrahydrocannabivarin (THV) and cannabivarin (CBV) respectively. Finally, cannabichromene (CBch) was the least travelled extract.

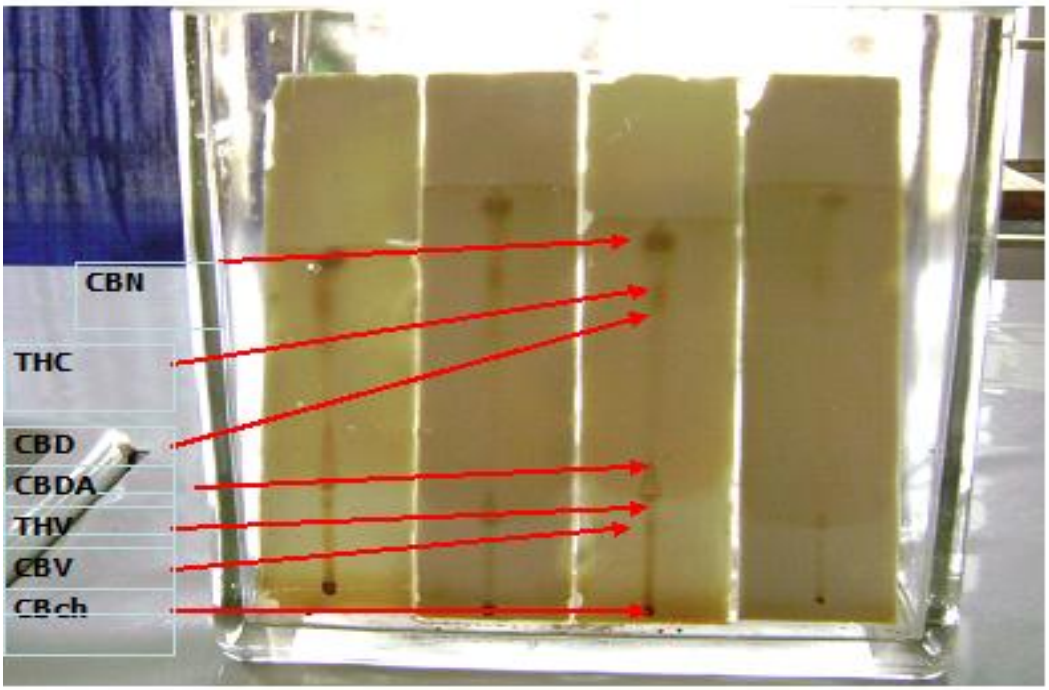

Plate 1: Thin Layer Chromatography (TLC) Column of Cannabis sativa Leaf showing: Cannabinol (CBN); Tetrahydrocannabinol (THC); Cannabidiol (CBD); Cannabidioc Acid (CBDA); Tetrahydrocannabivarin (THV); Cannabivarin (CBV); and Cannabichromene (CBch)

Discussion

Phytochemical screening of *C. sativa* leaves of revealed the presence of alkaloids, flavonoids, cardiac glucosides, terpenes, resins and steroids. These compounds when present in animal's body have been reported to poison or sedate the animal.[22-24] Cardiac glucosides and alkaloids were reported to cause reduction in the blood glucose level of rat exposed to leaf extracts of *Vernonia amygdalina* while terpenes caused erratic pattern of swimming in fishes.[24, 25] Francis *et al*. reported that the presence of these compounds and their by products in fish systems interferes with food utilization and affect the general health of fish.[26] Although, Roy and Datta *et al*. reported that saponin is diversely distributed among the plant family, this compound was not found in the leaf extracts of *C. sativa*.[27]

The proximate composition of *C. sativa* leaves showed that the leaves contain low percentage (23.78%) crude protein. This is more than the lowest crude protein of 20% recommended in fish feedstuff.[28] Suggestive of the possibility of incorporating *C. sativa* leaves into the diets of adult teleost such as tilapia with a protein requirement of 25-35% recommended for grow-out tilapia.[29] There was wide margin observed with the proximate seed composition in this work and that reported of the seed oil content of *C. sativa* with protein, fibre, lipid, ash, moisture and carbohydrate contents of 23.90, 17.30, 32.21, 4.32, 3.07 and 28.50% respectively.[30]

Proteins are basically composed of amino acids as building blocks and fishes have been reported to require ten essential amino acids (EAA) in their diets which are species dependent. According to Mambrini and Guillaume et al., all teleosts require the following ten essential amino acids namely: Lysine, Histidine, Arginine, Threonine, Valine, Methionine, Isoleucine, Leucine, Phenylalanine and tryptophan.[31] This assertion was further echoed by Mambrini and Kauskik *et al.*[31] on the deficiency of Methionine to salmonds; Rodehutscord *et al.*[32] on growth retardation, poor feed efficiency, resistance to diseases and lower immune responses due to deficiency of indispensible amino acids and enhanced growth performance due to tryptophan inclusion in the diet of juvenile Asian sea bass.

In this research, nine (9) of the ten EAA namely Lysine, Histidine, Arginine, Threonine, Valine, Methionine, Isoleucine, Leucine, and Phenylalanine were found in good quantity in the leaves of *C. sativa*. The amino acid profile of *C. sativa* showed that the leaf has comparable amino acid contents to those from egg white and soybeans in quality and those obtained from hempseed.[33, 34] The fact that trypsin inhibitory substances are absent in cannabis protein, partially explains the superiority of cannabis protein to soybean.[35] The amino acid profile obtained in this study could provide baseline information of cannabis as protein source for supplementing fish protein requirements. However, this must been done with caution since plant protein sources are poorer than fishmeal which contain some amino acids not present in the tissues of terrestrial plants.[36]

The TLC column obtained in this investigation agreed with the earlier findings of Perkin-Elmer Corp.[20] This corporation revealed that six to seven bands are formed depending on temperature, chamber saturation and other environmental conditions that might have influence the growth of the C. sativa plant. They opined that, the highest travelled band is cannabinol (CBN) closely followed by tetrahydrocannabinol (THC), cannabidiol (CBD), cannabidioc acid (CBDA), tetrahydrocannabivarin (THV), cannabivarin (CBV), and finally, cannabichromene (CBch). On characterization, the authors obtained a refractive index (Rf) value of 0.55. Similar results were reported by Baker *et al*. who also showed that many factors such as genetic characteristics of the seed stock, the environment in which the plant was grown, maturity, sex and part of the harvested plant and the time which has elapsed between harvesting and chemical analysis as well as the conditions of storage affects the plant ingredients content.[37] In a related study Shobha reported Rf value of 0.89 of *C. sativa* seeds at 40ºC.[30] Little doubt that the leaf cannabinol Rf value obtained in this work (Rf 0.50) did not agree with the standard value of Rf 0.55 mainly due to the type of storage the drug received as a contraband meant for destruction by the NDLEA of Nigeria.[20, 37]

Conclusion

In conclusion, phytochemical analysis revealed important bioactive compounds that can reduce stress conditions, boost appetite and immunity and seduce fish. Others compounds obtained through the proximate analysis showed that the plant has the potentials to partially supplement protein requirement of warm water fishes, besides, the good array of EAA obtained in this research can form baseline information for possible utilization of this cheap, readily grown and accessible weed in fish nutrition thereby ameliorating the overall cost of fish production in artificial confinements.

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