Taxonomic Authentication of Two Morphologically Identical Senna Species Using Matk DNA Barcoding and Phytochemical Protocol

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Abstract

Taxonomic judgments based solely on morphological characters have been found to be problematic and unfair, especially when more reliable tools such as DNA barcoding exist to establish and discriminate between taxa. In this study, the chloroplast DNA region (matk) of *S. Bicapsularis* and *S.sulfurea* were studied. The objective was to evaluate its applicability in discriminating among these morphologically similar species. DNA samples obtained from the species were studied in terms of DNA purity, PCR amplification, amplicons nucleotide, protein sequences, sequence authentication and phylogeny. The results showed similarities among the two species with respect to DNA purity, PCR amplification but revealed obvious differences between their amplicons in terms of sequence characteristics, homology results and phylogeny. The nucleotide sequence of *S. sulfurea* and *S. bicapsularis* had 364 and 685 base pairs respectively, with the corresponding 121 and 228 amino acids residue. Sequences of both species were unambiguously identified with 97.25 and 99.55 % identities with *S. sulfurea* and *S. bicapsularis* respectively. A total of 393 SNPs, corresponding to 106 mutated amino acid residues were recorded between the evaluated species; representing 40.9 and 52.2 % nucleotide and amino acids sequences. An evolutionary distance of 1.4 % was recorded between the studied sequence showing that *S. sulfurea* and *S. Bicapsularis* are separate taxa, while a strong bootstrap value of 99 % depicted a reliable evolutionary relationship and confirmed their placement in same genus. To ensure species authentication in future without recourse to molecular study, routine phytochemical tests were performed on the species to determine reaction specificity. From the eleven tests conducted, flavonoid, triterpene and steroids/phytosterols showed differential colour changes, hence were effective in discriminating between both species upon which a taxonomic key was constructed.

Keywords: DNA barcoding, Taxon idenification, Phytochemistry, Species discrimination, Sequence authentication.

1.0 Introduction

Much conflicting reports and arguments exist in the scientific world on the taxonomic nomenclature used by most researchers across regions of the world with poor repository of plant DNA information. The propensity for use of inaccurate nomenclature is particularly grave in areas with dearth of well-trained morpho-taxonomists and in climes such as the tropics with high rate of speciation (Bennett & Balick, 2008). The problem is further compounded by the continual use of ancient nomenclature of a particular species from time immemorial and which characteristics have changed over time due to evolutionary forces and the gap created by these changes, thus creating the need to seek expert knowledge in authenticating the research plant materials of interest.

The absence of stringent regulations mandating all research voucher specimens to be rigorously authenticated and deposited in herbaria has fuelled this impunity with reckless abandon. The weight of the act on humanity and science if allowed to persist is grave. First, it endangers life, as a sizeable percentage of poorly educated rural and semi urban dwellers adopt and make use of published research findings and recommendations on herbal recipes. Their preference for published herbal products over unpublished traditional preparations stems in part from the well documented ills of the latter (Li-Weber, 2009). Secondly, it erodes science in these climes of its premium on step wise methodological approaches and bequeaths an unscientific culture to the upcoming generations. It also erodes confidence when significant differences are obtained. Active ingredients or bioactive components on further analyses using the actual plant material bearing the accurate name is subjected to further scientific analyses in other climes.

The ultimate casualty is the society, where graduates become teachers and industry experts who will pass on this inaccurate scientific name and culture of the wrong taxon to successive generations. The good thing is that this challenge can be solved. DNA fingerprints are taxon-specific. Compilation of DNA data bank for morphological identical species using stored information from NCBI and other recognized species in Gene bank would bond such taxon to its accurate nomenclature (Kader &Chellakumar, 2015). For consumption of the wider audience and to ensure future researchers do not need to conduct DNA analyses to ascribe an accurate name to a taxon, basic laboratory phytochemical tests need to be conducted on the plant materials whose accurate name has been determined using DNA analyses to observe and document trends for taxonomic key construction (Mishra et al, 2016). The follow up phytochemical profiling and authentication process must be less expensive, less elaborate, user friendly, repeatable, reproducible and time saving to achieve purpose.

*Sennabicapsularis (*Aidantoro *)* and *Sennasulfurea* (Aidantoro) are two distinct species that are morphologically almost inseparable. Several herbaria and curators in Nigeria often assign the better known *Sennaalata* and in few instances *Sennatora* to other lesser known Senna species (personal communication and observations) (Soladoye*et al*, 2010). This is partly exemplified by the ratio of research works on *S.bicapsularis* and *S.sulfurea* species to that of *Sennaalata* and *Sennatora*. It is in the light of these challenges that the study aims at firstly employing the

technical, not readily available and expensive DNA marker to discriminate between the morphologically identical *S.bicapsularis* from *S.sulfurea* and secondly, to construct a taxonomic key for both species using the routine phytochemical test that can easily be applied in field studies and laboratories by undergraduates and curators for future and referential authentications.

2.0 Materials and Methods

2.1 Sample Collection

Five morphologically identical unknown specimens of *Senna* were obtained from the wild in Afi and Okwango Divisions of the Cross River National Park in August 2018 (wet season) and another five individuals from same stock in January 2019 (Dry Season). The essence was to observe influence of seasonal variations on the phytochemical signatures.

2.2 Sample Preparation

Each test sample was ground to powder after oven drying for 72 hours and stored in vials at room temperature. 500g of each was ground, sample was reserved for DNA analysis and another 500g were reserved for phytochemical analysis.

2.3 DNA extraction

Extraction was done using a ZR plant DNA mini prep extraction kit. Chopped pieces of plants leaves were transferred into a ZR Bashing Bead Lysis tubes. 750 microlitre of lysis solution was added to the tubes. The tubes were secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube was centrifuged at 10,000rpm.

Four hundred (400) microlitres of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7, 000rpm. One thousand two hundred (1, 200) microlitres of plant DNA binding buffer was added to the filtrate in the collection tubes, bringing the final volume to 1, 600 microlitres, 800 microliter was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 rpm, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliters of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000 rpm followed by the addition of 500 microlitres of plant DNA Wash Buffer and centrifuged at 10,000 rpm. The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube, 100 microlitres of DNA elution buffer was added to the column matrix and centrifuged at 10,000g microlitre for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degrees for polymerase chain reaction (PCR).

2.4 DNA Quantification

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The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitres of the extracted DNA were loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button. This was done at an optical density (OD) of 260/280 nanometer readings.

2.5 MatK gene Amplification

The MatK gene of the isolates were amplified using the RandomMatK-1RKIM-forward: 5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3' and MatK-3FKIM-reverse: 3'-CGTACAGTACTTTTGTGTTTACGAG -~~3~~5', primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microliters for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes, denaturation, 95ºC for 30 seconds, Annealing, 53ºC for 30 seconds, extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1 % agarose gel at 120 V for 15 minutes and visualized on a blue light Tran's illuminator.

2.6 Sequencing

Sequencing was done using the Big Dye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl Big Dye terminator v1.1/v3.1, 2.25 µl of 5 x Big Dye sequencing buffer, 10 µM Primer PCR primer, and 2-10 ng PCR template per 100 bp.

2.7 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit. Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using Clusta W. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

2.8 Phytochemical Analysis

2.81 Preparation of Extracts for Preliminary Phytochemical Test

Ten (10)g of the candidate plant powder was weighed using a chemical balance (110c), transferred to a rubber bottle and soaked with 150ml absolute ethanol and incubated for 24 Hrs. for maximum extraction and filtered, first through a Whatmann filter paper No. 41 and then through cotton wool to obtain a clear solution . The solutions were stored and used for qualitative test.

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2.8.2 Phytochemical Test

A preliminary phytochemical test was conducted on the candidate plant extracts following the standard methods for phytochemical screening described by Usman, *et al* (2009). The resultant colour change, formation of precipitates, and foam, characteristic smell and time required for colour change in the extracts were observed and noted. The tests conducted were reviewed to be present in the genus to which the candidate species belong.

Table 1. : Basic Phytochemical tests on *S. sulfurea* and *S bicapsularis*

Test	Procedure
Flavonoids (Basic)	Three (3) ml of extracts were treated with 3 drops of aqueous NaOH followed by 3 drops of 40% HCl and observed for changes. Usman,*et al* (2009).
Coumarins	Three (3) ml of the extract was treated with 3 ml of 10% NaOH and observed for changes
Flavonoids (Acidic)	2 ml of plant extract was treated with 4 drops of concentrated sulphuric acid and observed for changes
Tannins	To 1 ml of plant extract, 2 ml of 5 % ferric chloride was added (Iyengar, 1995).
Phenols	To 1 ml of the extract, 2 ml of distilled water followed by 5 drops of 10% ferric chloride was added and observed for changes
Anthocyanins	To 4 ml of extract was treated with 2 ml of HCl followed by 2 ml of Ammonia and observed for changes
Glycosides	To 2 ml of plant extract, 1 ml of glacial acetic acid and 5% ferric chloride was added, 3 drops of concentrated sulphuric acid was added and observed for colour change (Grease and Evans, 1989).
Triterpenes	Chloroform solution of the extract with few drops of acetic acid and one ml concentrated sulphuric acid
Diterpenes	To 2 ml of extracts, 1 ml of aqueous solution of copper acetate was added and observed for colour change
Steroids&phytosterols	To 1 ml of plant extract, equal volume of chloroform and 3 drops of concentrated sulphuric acid were added and colour changes were noted
Saponins	To 1 ml of plant extract, 5-10 ml of distilled water was added and shaken for 2 min observing the formation of foams (Bushra et al, 2018).TableauColour Palettes was used for observation

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3.0 Results and Discussions

3.1 Matk Gene Status of the Study Taxa

Results showed that the total DNA purity index of samples A and B were 1.06 and 1.16 respectively. The *Matk* gene amplification was successful in both samples. Plate 1 shows the amplification (PCR) results of the studied samples relative to a 1 kb ladder. Table 1 outlines the *matK* gene features of the studied samples. Table 2 presents information on sequence variation between the two samples, while Fig. 1 is a Neighbor-Joining tree depicting the phylogenetic relationships between the studied sequences and their identical sequences from NCBI database.

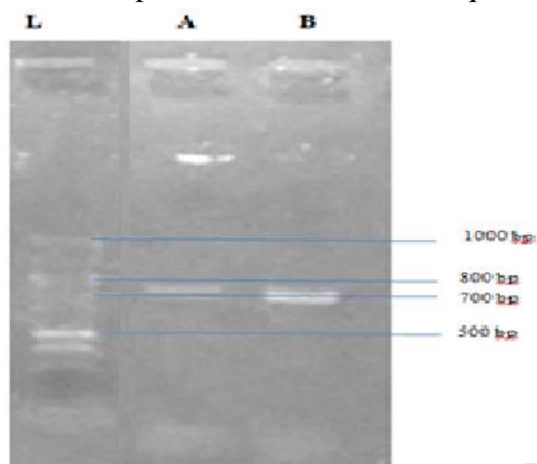


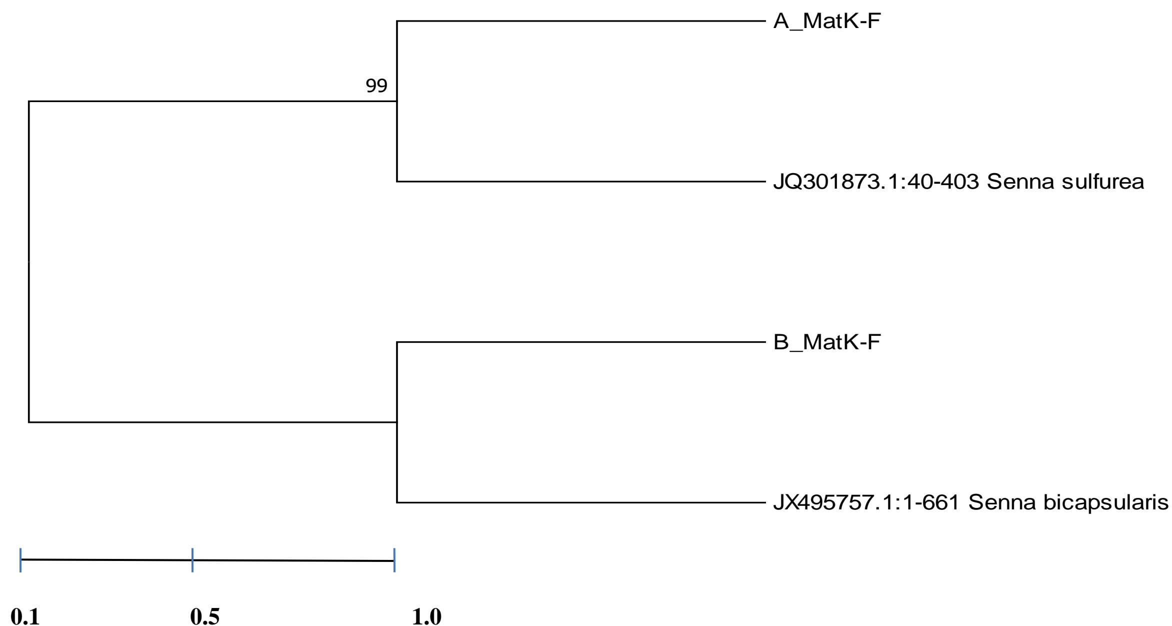
Plate 1: DNA Amplification results

Table 2: DNA characterization of samples A and B using *matK*gene sequences

Characteristic	Sample A	Sample B
DNA purity ($A_{260/280}$)	1.06	1.16
PCR reading relative to the ladder	650 – 750 bp	650 – 750 bp
Nucleotide sequence length	364	685
Amino acid sequence length	121	228
BLAST similarity (%)	97.25	99.55
NCBI most identical sequence	*S. sulfurea*	*S. bicapsularis*

Table 3: Sequence variability between samples A and B

Characteristic	Nucleotide sequence	Amino acid sequence
Conserved regions	280	119
Point mutation	4	2
Indel mutation	389	104
Unsequenced/untranscribed regions	12	13
Sequence similarity (%)	40.9	52.2
Evolutional distance	0.014	0.014



0.1 0.5 1.0

Figure 1: Phylogenetic tree showing evolutionary relationship between the matk gene barcode and the test taxa

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DNA of the two (2) morphologically similar species of *Senna* was extracted for sequencing at the *matK* gene region. The result obtained showed that the DNA solution of both samples A and B were below the purity mark of 1.8 (Oxford Gene Technology, 2011). This suggests the presence of protein contaminants in the DNA isolate of these samples (Abdel-Latif and Osman, 2017). The intense absorption is primarily due to the presence of aromatic rings in the purine, pyrimidine (Sathyamurthy*et al.*, 2019). The DNA quality recorded in the present study did not vary significantly and therefore could not be used as a distinctive character in these species.

The *matK* gene fragment of the sample's DNA was amplified using universal primer set. Result showed that both samples were successfully amplified, indicating a 100 % amplification success. Previous researches suggest that matK PCR success rate is highly variable, ranging from 40% to 97% (Kress and Erickson, 2007; Nithaniyal*et al*., 2014). The amplicons in the present study were found in the region of 650 – 750 base pairs corresponding to the ladder (Plate 1). The DNA bands were bold and sharp proving that the *matK* genes of the studied DNA samples were intact and could be used for sequencing. The present findings compete favourably with those of Ha *et al*. (2018) and Nithaniyal *et al*. (2014), who reported 750bp and 607bp respectively with *matk* region in their studies.

The bidirectional sequencing was successful in both samples representing 100 % success. The obtained nucleotide sequences of samples A and B had 364 and 685bp respectively while the

amino acids sequences had 121 and 228 residues respectively. Nithaniyal *et al*. (2014) reported sequence length that varied between 508 bp and 867 bp with an average of 803 bp and upheld that 500 bp is acceptable for the submission to BOLD database. According to Kress *et al*. (2005), sequence length of 300–800 bp satisfied the criterion of an appropriately short sequence length to facilitate DNA extraction and amplification. Therefore, the *matK* gene of the samples A and B adequately satisfied the requirement to be used as barcode region.

Organisms with high percentage sequence similarity in their genes have a similar pattern of evolution and differentiation (Stone *et al.,* 2010). According to Kajita *et al.* (2001), if two sequences have sequence identity greater than 70%, it implies that they have about 90% probability or more to share the same biological processes and functions. It was found ~~out~~ from the present study that nucleotide and amino acid sequence identity was less than 70 % for samples A and B. This implies distant relationship and might suggests that the *matK* gene found in samples A and B may perform different functions and undergo the dissimilar processes (Udensi *et al.,* 2017).

The identity between the query and the reference sequences is the basic principle of molecular identification and phylogenetic reconstruction (Wattoo*et al*., 2016). The matK sequence of samples A and B in this study were identified to belong to *Sennasulfurea* and *Sennabicapsularis* with the 97.25 and 99.55 % identities respectively. Their identity with these taxa was unambiguous (Shinwari *et al.,* 2014). The evolutional distance of 0.0 % was regarded between the samples A and B and *Sennasulfurea* and *Sennabicapsularis* respectively, indicating the absence of barcode gaps and close homology (Trina *et al.,* 2016).

The evolutional distance of 1.4 % was recorded between samples A and B, this satisfied the requirement for species differentiation using a single DNA marker (Tallei and Kolondam, 2015; Purushothaman *et al*. 2014). This indicates the availability of barcode gaps between the studied sequences. According to Trina *et al.*, an ideal DNA barcode should exhibit a barcoding gap between inter-specific divergence and intra-specific distance. The amount of barcode gaps between sequences is qualitatively related to the sequences' evolutionary distance from one another. Thus, high sequence variation indicates high barcode gaps, suggesting that the sequences have a comparatively young most recent common ancestor. From the foregoing, it could be said that samples A and B are sister taxa (Kang *et al*., 2017).

Phylogenetic tree analysis (Fig. 1) equally revealed that samples A and B belong to *Sennasulfurea* and *Sennabicapsularis* respectively with strong bootstrap values of 99 % respectively, indicating highly reliable evolutionary relationships for the studied *senna* species (Kang *et al*., 2017). This means that *matk* can be used as barcode region for phylogenetic reconstruction for the studytaxa.

3.3 Phytochemical analysis and profiling of study taxa

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The result of various basic phytochemical screening tests meant forinsitu authentication of the two species as shown in Table 4.

Table 4: Results of Phytochemical tests on plant samples

S/N	Tests	Species	Initial colour	Final colour	Duration for colour change (Secs.)	Remarks
1	Flavonoid (basic)	*S. sulfurea* *S. bicapsularis*	Green	Orange yellow for both species	5 and 8 respectively	Do not discriminate between test species
2	Coumarins	*S. sulfurea* *S. bicapsularis*	Green	Dark green for both species	4 for both species	Do not discriminate between test species
3	Flavonoids (acidic)	*S. sulfurea* *S. bicapsularis*	Green	Orange and dark brown respectively	2 and 3 seconds respectively	Revealed discriminatory results between testspecies
4	Tannin	*S. sulfurea* *S. bicapsularis*	Green	Dark blue to greenish black for both species	2 for both species	Do not discriminate between test species
5	Phenol	*S. sulfurea* *S. bicapsularis*	Green	Light green for both species	3 and 2 respectively	Do not discriminate between test species
6	Anthocyanin	*S. sulfurea* *S. bicapsularis*	Green	Dark brown for both species	8 sec for both species	Do not discriminate between test species
7	Glycoside	*S. sulfurea* *S. bicapsularis*	Green	Dark brown for both species	4 sec for both species	Do not discriminate between test species
8	Tri-terpene	*S. sulfurea* *S. bicapsularis*	Green	Deep red colours between two layers and dark brown respectively	2 min and 3 min respectively	Revealed discriminatory results between test species
9	Diterepene	*S. sulfurea* *S. bicapsularis*	Green	Sea green for both species	2 sec for both species	Do not discriminate between the test species
10	Steroids and phytosterols	*S. sulfurea* *S. bicapsularis*	Green	Brown rings and dark green	5 sec and 8 sec respectively	Revealed discriminatory results between test species
11	Saponin	*S. sulfurea* *S. bicapsularis*	Green	-	-	Do not discriminate between test species

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A taxonomic key is constructed based on results shown in Table 1 as shown in Fig 2.

1a Specimen colouration changes from Green to Orange within 2 seconds when exposed to acidic flavonoid test*S. sulfurea*

1b Specimen colouration changes from Green to Dark brown within 3 seconds when exposed to acidic flavonoid test. *S. bicapsularis*

2a Specimen colouration changes from Green to deep red between two layers within 2 minutes when exposed to Tritepenoid test. *S. sulfurea*

2b Specimen colouration changes from Green to Dark-brown within 3 minutes when exposed to Tritepenoid test. *S. bicapsularis*

3a Specimen colouration changes from Green to Brown rings within 5 seconds when exposed to Steroids and phytosterols test. *S. sulfurea*

3b Specimen colouration changes from Green todark green within8 seconds when exposed to Steroids and phytosterols test. *S. bicapsularis*

Figure 2: Taxonomic key for authenticating *Sennasulfurea*from *Sennabicapsularis*

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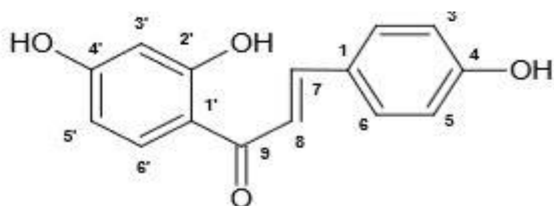
The ability of flavonoids, sterols/phytosterils and triterpenes to discriminate between both species could be owed to the presence of isoliquiritigenin and chrysin(flavonoids), in *S. sulfurea* and its absence in *S. bicapsularis*. It could also be due to the presence of 4-methoxy-α-L-xylopyranosyl-(3→1′)-O-α-L-4′-methoxyxylopyranoside (di-4-methoxy-α-L-xyloside) a phytosterils in *S. sulfurea* and its absent in *S bicapsularis*(Sultana*et al* 2018). The dark green colouration observe for the steroid test of *S bicapsularis*shows the presence of Cholesterol, which is absent in *S. sulfurea.* The structure of these compounds as shown in Fig 3.



(a)

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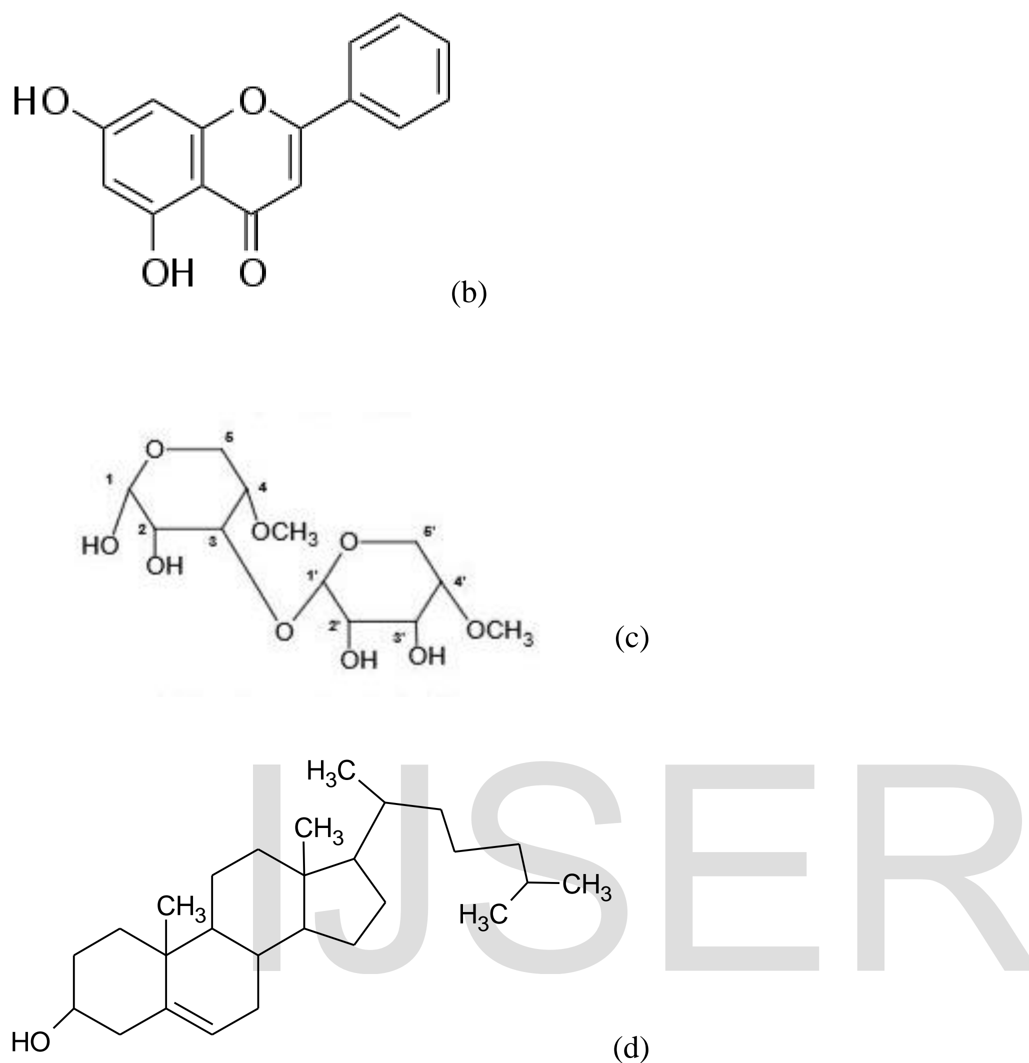

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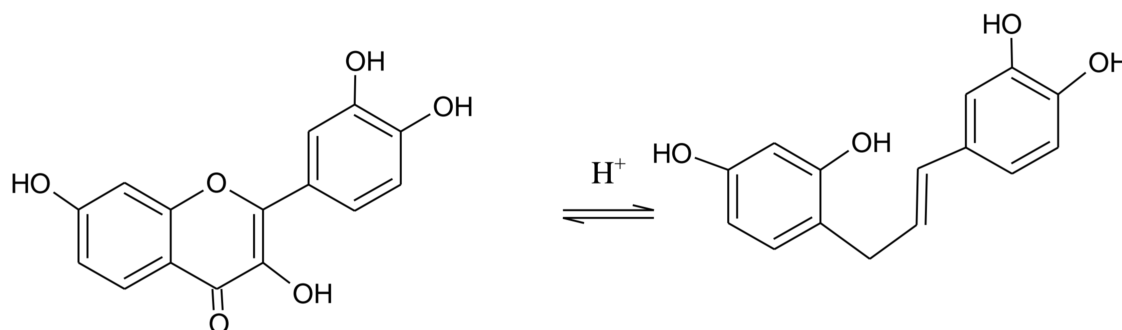
Fig 3: Chemical constituents present in the leaves of *S.sulfurea*and absent in *S.bicapsularis*(a) Isoliquiritigenin, (b) Chrysin, (c) Di-4 methoxy- α-L-xyloside, (d) Cholesterol

Isoliquiritigenin belongs to the group of flavonoids referred to as Chalcones. Isoliquiritigenin and Chrysin are highly conjugated and hydroxylatedflavones. There exist pigments in most chalcones, hydrochalcones and aurones which, under chemical processes, change from yellowish green to Orange (Evranos-Aksoz and Ertan, 2011). This colour change can be attributed basically to the highly conjugated structure of Isoliquiritigenin. Also, the chromophores present improve light absorption wavelength. The ethanol extract of *S. sulfurea*on treatment will experience pi-electron delocalization, which originated from the ionization of hydroxyl groups of Isoliquiritigenin and chrysinstructures. This enhanced light absorption wavelength to about 595–605 nm (orange color zone of visible rays of the electromagnetic spectrum) (Andre *et al,* 2007).

De-epoxidation occurs in flavonoids on treatment with sulphuric acid to give a reddish orange product (Harbone, 1984), as shown in Fig 4. This supports the Flavonoid test results (positive) for *S. sulfurea,* where the initial green solution turned orange upon addition of sulphuric acid. 2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-4*H*-chromen-4-one, a hydroxylated derivative of chrysin is de-epoxidized to 4-[(1*E*)-3-(2,4-dihydroxyphenyl)prop-1-en-1-yl]benzene-1,2-diol (Reddish).



2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-4*H*-chromen-4-one4-[(1*E*)-3-(2,4-dihydroxyphenyl)prop-1-en-1-yl]benzene-1,2-diol

Figure 4. De-epoxidization of 2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-4*H*-chromen-4-one, to give a Reddish product.

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Treatment of phytosterols/cholesterol with sulphuric acid gives a dark bluish green colouration (Weny et al, 2019). This dark green colour is achieved due to the hydroxyl group (-OH) of cholesterol reacting with the sulphuric acid reagent, hence increasing the degree conjugation of the unsaturation in the adjacent fused ring (Sabir et al, 2003). This reaction generates cholesta-3,5-diene which exhibits a dark green colour.

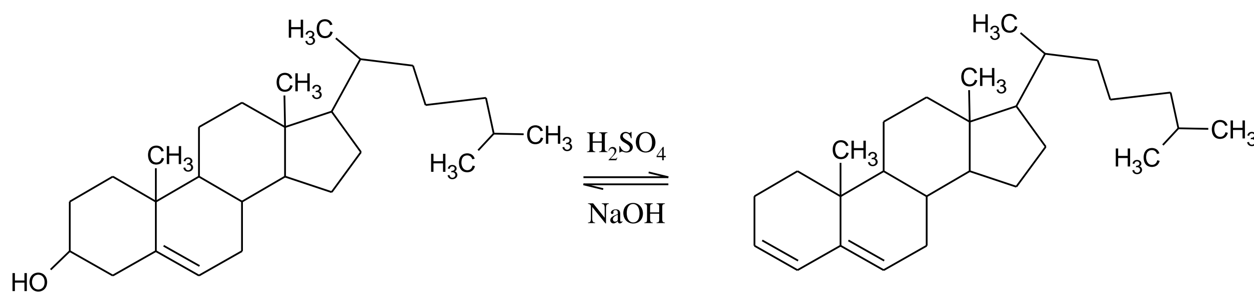

Figure 5. Reaction of Phytosterol/Cholesterol to give a dark green Cholestadiene

The test for triterpenes yielded dark brown and deep red colours for *S. sulfurea* and *S. Bicapsularis* respectively. A positive test for a chloroform solution of triterpenes, while using acetic acid and sulphuric acid is expected to gives a deep blue system, with fluorescent in the chloroform layer (Ishida et al, 1999). Hence, no definite triterpene is confirmed for both *Senna* species investigated.

4.0 Conclusion

The result of the study showed that *matk* gene can be used as barcode region for authentication and phylogenetic reconstruction for *Senna* species and the samples studied belong to separate species (*S. sulfurea* and *S. Bicapsularis* respectively) despite their identical morphometrics. The routine use of phytochemical protocols proved patents that could be employed subsequently without recourse to molecular study.

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