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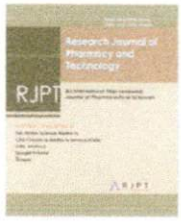
Volume - 1
Volume - 2
Volume - 3
Volume - 4
Volume - 5
Volume - 6
Volume - 7
Volume - 8
Volume - 9
Volume - 10
Volume - 11
Issue - 1
Issue - 2
Issue - 3
Issue - 4
Issue - 5
Issue - 6
Issue - 7
Issue - 8
Issue - 9
Issue - 10
Issue - 11
Issue - 12
Volume - 12
Volume - 13



Volume - 11 | Issue - 12
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Determination of Antioxidant Capacity, Flavonoids and Total Phenolic Content of Extracts from Atractylis flava Desf (AbstractView.aspx?PID=2018-11-12-1)

Mohamed Akram Melakhessou, Naima Benkiki, Salah Eddine Marref
DOI: *10.5958/0974-360X.2018.00952.6*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-1) [Cite] Viewed: 1 (pdf), 648 (html) Private Access

Application of Response Surface Methodology in Process Parameter Optimization of Media for Production of Amylase (AbstractView.aspx?PID=2018-11-12-10)

R. Subbaiya, S. Priyanka, D. Suresh, M. Masilamani Selvam, R. Balachandar S. Chozhavendhan
DOI: *10.5958/0974-360X.2018.00961.7*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-10) [Cite] Viewed: 1 (pdf), 480 (html) Private Access

Biosynthesis and Characterization of Silver Nanoparticles from Aspergillus niger and its Antibacterial Activity (AbstractView.aspx?PID=2018-11-12-11)

Suresh Dhanaraj, P. Bharathiraja, R. Dhandapani , R. Subbaiya, A. K. Kathiresha
DOI: *10.5958/0974-360X.2018.00962.9*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-11) [Cite] Viewed: 1 (pdf), 372 (html) Private Access

A Retrospective Study on prescribing pattern of drugs among pregnant inpatients in Tertiary Care Hospital (AbstractView.aspx?PID=2018-11-12-12)

Sri Roja. I, Shanmugasundaram. P, Sathesh Kumar. S
DOI: *10.5958/0974-360X.2018.00963.0*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-12) [Cite] Viewed: 1 (pdf), 341 (html) Private Access

Cardioprotective Activity of Telmisartan, Metformin and its Combination against Doxorubicin Induced Myocardial Infarction in Rat Model (AbstractView.aspx?PID=2018-11-12-13)

P. Aruna, N. M. Gayathiri
DOI: *10.5958/0974-360X.2018.00964.2*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-13) [Cite] Viewed: 1 (pdf), 338 (html) Private Access

1-Eicosane, A Hydrocarbon from Curvularia lunata an endophytic fungi isolated from bark tissue of Ficus religiosa (AbstractView.aspx?PID=2018-11-12-14)

Sundaramoorthy Maheshwari, Kalyanaraman Rajagopal, Vaithianathan Sriraman, Kumar Lokesh, Meenambiga S. S., Meenashree Balakrishnan, Arulmathi Ramalingam
DOI: *10.5958/0974-360X.2018.00965.4*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-14) [Cite] Viewed: 1 (pdf), 372 (html) Private Access

Dynamics Persistent Potential of Bacteria as a Treatment Target in Pyoinflammatory Diseases (AbstractView.aspx?PID=2018-11-12-15)

Zaira F. Kharaeva, Magomet Sh. Mustafaev, Larisa Z. Blieva, Ibragim V. Hulaev, Oksana M. Gendugova, Djamilya S. Bozieva
DOI: *10.5958/0974-360X.2018.00966.6*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-15) [Cite] Viewed: 1 (pdf), 378 (html) Private Access

Formulation and Evaluation of Pioglitazone HCl Fast Dissolving Tablet using Solid Dispersion (AbstractView.aspx?PID=2018-11-12-16)

Pinky Yadav, Pankaj Patel, Prabhat Das, Nitin Deshmukh, Sujit Pillai
DOI: *10.5958/0974-360X.2018.00967.8*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-16) [Cite] Viewed: 1 (pdf), 371 (html) Private Access

D Hypovitaminosis in Syrian Type 2 Diabetes Mellitus Patients: Lack of Relationship with Glycemic Control (AbstractView.aspx?PID=2018-11-12-17)

Razan Ibrahim, Mohammad Imad Khayat, Afraa Zrieki
DOI: *10.5958/0974-360X.2018.00968.X*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-17) [Cite] Viewed: 1 (pdf), 348 (html) Private Access

Extraction, Isolation and Analysis of Chondroitin Sulphate from Chicken Shank by Spectrophotometric Method (AbstractView.aspx?PID=2018-11-12-18)

Rizky Amalia Adlina Affandhi, Juni Ekowati, Noor Erma Nasution Sugijanto
DOI: *10.5958/0974-360X.2018.00969.1*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-18) [Cite] Viewed: 1 (pdf), 416 (html) Private Access

The Development of Identification and Quantitative Definition Methodic of Active substances of the Ointment "Allergolic" (AbstractView.aspx?PID=2018-11-12-19)

Rukhmakova Olga, Yarnykh Tatyana, Tykhonov Olexander
DOI: *10.5958/0974-360X.2018.00970.8*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-19) [Cite] Viewed: 1 (pdf), 319 (html) Private Access

Effect of Stress Inducer on the Morphology of the Riboflavin producer Eremothecium ashbyii (AbstractView.aspx?PID=2018-11-12-2)

Sampath Kumar Jadhav, S. Vijayalakshmi
DOI: *10.5958/0974-360X.2018.00953.8*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-2) [Cite] Viewed: 1 (pdf), 343 (html) Private Access

Association of Neutrophil/Lymphocyte (N/L) Ratio and Spatial and Verbal Memory in Females (AbstractView.aspx?PID=2018-11-12-20)

Sahithi Alaghari, Kumar Sai Sailesh
DOI: *10.5958/0974-360X.2018.00971.X*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-20) [Cite] Viewed: 1 (pdf), 336 (html) Private Access

Effectiveness of Eccentric Strengthening of Wrist Extensors along with Conventional Therapy in Patients with Lateral Epicondylitis (AbstractView.aspx?PID=2018-11-12-21)

Anitha. A, Prachi. G
DOI: *10.5958/0974-360X.2018.00972.1*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-21) [Cite] Viewed: 1 (pdf), 315 (html) Private Access

In vitro Anti-Cancer Study of Vitis Viniferae, Ixora Coccinea and Piper Longum Ethanolic extracts on Human Breast Carcinoma Cells (AbstractView.aspx?PID=2018-11-12-22)

Deeparani K. Urolagin, S. Jayakumari
DOI: *10.5958/0974-360X.2018.00973.3*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-22) [Cite] Viewed: 1 (pdf), 471 (html) Private Access

Development and Evaluation of Sustain Release Pioglitazone Microsphere (AbstractView.aspx?PID=2018-11-12-23)

Anand Patel, C. C. Patil, R. V. Kulkarni, Vijaykumar A, Prashant Jorapur, Akshay Deshpande, Swapna W
DOI: *10.5958/0974-360X.2018.00974.5*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-23) [Cite] Viewed: 1 (pdf), 311 (html) Private Access

HPTLC Fingerprinting Analysis of Tannin Profile on Canthium coromandelicum and Flueggea leucopyrus willd. (AbstractView.aspx?PID=2018-11-12-24)

A. Anto Arockia Raj, J. Vinnarasi R. Venkataraman, M. Augustin
DOI: *10.5958/0974-360X.2018.00975.7*
[Abstract] [PDF Paper] [HTML Paper] (HTMLPaper.aspx?Journal=Research Journal of Pharmacy and Technology;PID=2018-11-12-24) [Cite] Viewed: 1 (pdf), 327 (html) Private Access

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RESEARCH ARTICLE

Extraction, Isolation and Analysis of Chondroitin Sulphate from Chicken Shank by Spectrophotometric Method

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ABSTRACT:

This study evaluated Chondroitin sulphate isolated from chicken shank cartilage in aspects such as sufficient quantities and requisite quality for feasible source of Chondroitin sulphate. Chondroitin sulphate has been widely used in treatment of osteoarthritis. Chondroitin sulphate not only reduces joint pain, but also inhibiting the disease progression. Chondroitin sulphate was obtained from cartilage of chicken shank, using enzyme (papain), acid (acetic acid), and heat. The cartilage obtained from chicken shank was hydrolysed using papain for 24h. Extraction was carried out using acetic acid and incubated at pH 4.5 and 37°C for 6h and 8h. The supernatan was treated with cetylpyridinium chloride (CPC), NaCl, methanol and ethanol. The precipitate obtained from extraction was dried in oven at 55°C.Chondroitin sulphate yield from 6h incubation was 16.30% and from 8h incubation was 36.35 % (dry weight). The FT-IR spectra of extracted Chondroitin sulphate was identical with Chondroitin sulphate standard (Fenchem Biotek Ltd). Overlay of the spectras showed characteristic absorption at the wave number 854.64 cm^{-1} for sample and 856.54 cm^{-1} for standard chondroitin sulphate. Quantitative analysis was performed using spectrophotometry. The precision was good with RSD <7.3% and the accuration was good with recovery of 82.48-108.88%.The detection limit and quantification limit were 0.15 ppm and 0.46 ppm. This study shows that chicken shank cartilage produces a source for isolation of chondroitin sulphate of 36.35% (dry weight) and purity of 87.87%.

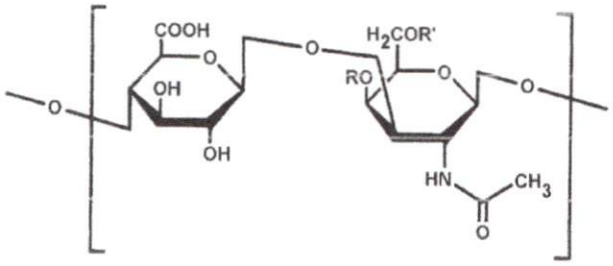
KEYWORDS: Chondroitin sulphate, chicken shank, spectrophotometric metodh, isolation, extraction.

INTRODUCTION:

Chondroitin sulphate (CS) is a glycosaminoglycan (GAG) comprised of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid.[1] Chondroitin sulphate has been widely used in treatment of osteoarthritis. Osteoarthritis is a progressive disease of whole joint and involves subchondral bone destruction, formation of osteophytes (bone spurs), synovial inflammation and cartilage loss (ref). One of the risk factors is age, where the disease increases with age. Osteoarthritis has also been associated with a reduced quality of life and increased healthcare costs.[2] Osteoarthritis affects Indonesians in which over 5% population aged below 40 year-old, 30% in age between 40 and 60 year-old, and 65% in age above 61 year-old. The prevalence of knee osteoarthritis is quite high with 15.5% for men and 12.7% for women.[3] Chondroitin sulphates is Symptomatic Slow-Acting Drugs in Osteoarthritis (SYSADOAs) and not only reduces joint pain, but also inhibiting the disease progression.[2]

Chondroitin sulphate occurs naturally in the extracellular matrix of connective tissues, e.g., bone, cartilage, skin, ligaments and tendons. Commercially available chondroitin sulphate is extracted from animal cartilage such shark and then purified. However, extraction process may results in degradation such as reducing molecular weight from 50–100 kDa to around 10–40 kDa (ref). To minimize chemical and structural modifications during extraction, a selective and robust extraction process should be used; while potent, non-specific oxidation in alkaline conditions and high temperatures should be avoided. Extraction should include enrichment, purification and solvent fractioning steps to produce chondroitin sulphate with a narrow

molecular weight range. Purification protocols are important to minimize contaminants, which can include other glycosaminoglycans, proteins, small organic molecules, and solvents [2]. The Structures of chondroitin sulphate (Figure 1).



Chondroitin sulfate A R = SO_3H R' = H
Chondroitin sulfate C R = H R' = SO_3H

Figure 1: Structures of chondroitin sulphate.

As an essential component of shark cartilage, chondroitin sulphate is widely used as a dietary supplement. Sharks are one of the most threatened groups of marine animals, as high exploitation rates coupled with low resilience to fishing pressure have resulted in population declines worldwide.[4] The other known sources of chondroitinsulphate used in nutritional supplements are the cartilaginous rings of bovine trachea and pork ears.[5] Nowadays chondroitin sulphate is produced only from porcine- trachea because of the risk of bovine spongiphorm encephalopathy (BSE). However, chondroitin sulphate which is produced from porcine-trachea cannot be consumed by muslim community. Popularity of CS and its limited sources makes CS a prime candidate for economic adulteration.[6] Because of its high price and limited sources, exploration on new sources of chondoroitin sufate which is easily obtained, sustainable and available with low cost are highly needed.

One of the biggest wastes of chicken farm is the shanks. Indonesian statistic reported that from 2003 there were 1.297.333.333 chicken shanks were produced from chicken farm waste. Chicken shanks's potency has not been optimally explored. Chicken shank is only used for making soup or another food and even ends up in trash-can.[7]

There are many isolation methods of chondroitin sulphate including alkaline hydrolysis, acid hydrolysis enzymatic hydrolysis, and purification using gel chromatography.[5,8,9] The obtained CS was identified using Fourier Transform – Infrared (FT-IR) and quantitative analyzed by spectrophotometric method.

MATERIAL AND METHODS:

Chondroitin sulphate standard was obtained from Fenchem Biotek Ltd (China). The chicken shanks was obtained from market (Surabaya, Indonesia). Cetylpyridinium chloride (RonaCare® CPC), acetic acid, NaCl, methanol and ethanol, Ferric chloride, Hydrochloric acid and Potassium bromide were purchased from Merck (Darmstadt, Germany). Papain (food grade), resorcinol(pharmaceutical grade) and double distilled water were purchased from P.T. Brataco (Surabaya, Indonesia).

Isolation of chondroitin sulphate from chicken shanks:

The cartilage from chicken shank was separated from the hard bone, skin, and the flesh by boiling the chicken shank in water at 90-95°C for ten minutes. The cartilage obtained from chicken shank was hydrolysed by papain (50 g/550g cartilage) for 24 h and dried with oven at 55°C. The dried cartilage was chopped and ground in blender.[10] The cartilage in approximately 5 g, were used for each experiment. The cartilage was divided into two groups. First group was extracted by incubation at pH 4.5 using 20 mL acetic acid 10% and temperature of 37°C for 6h. The second group was incubated for 8h. After incubation, the supernatant was separated from the residue. CPC (3%) in solution of 0.8 M NaCl was added to the supernatant and then centrifuged at 3600 rpm for 20 minutes, and temperature of 4°C to remove precipitates that contain another GAGs. Solution of 2 M NaCl (5 ml) was added to the supernatant followed by methanol at the same volume of supernatant. Furthermore, ethanol was added to the solution until the precipitation stop. The precipitate obtained from extraction was dried in oven at 55°C. All experiment were done intriplicate.

Characterization of Chondroitin Sulphate Fourier Transform – Infrared:

Fourier Transform Infrared Spectrometer using potassium bromide (KBr) pellet technique was employed to identify the types of chondroitin sulphate in the sample. Standard of chondroitin-4-sulphate and chondroitin-6-sulphate were analyzed and used as standard spectra. Dried chondroitin sulphate sample, approximately 2 mg, was mixed with dried potassium bromide powder (100–200 mg), then pressed into thin disc under hydraulic press and used as a sample for FTIR analysis. The spectrum was obtained at mid-infrared region).[5]

Spectrophotometric Method:

Reagent preparation:

Ferric chloride (75 mg) was dissolved in 50 mL of concentrated hydrochloric acid. Ethanol containing 200

mg of resorcinol (5 ml) was added to this solution and volume was made up to 50.0 ml with concentrated hydrochloric acid.[11]

Preparation of standard stock solution:
About 100 mg of chondroitin sulphate working reference standard of known purity (92.42% pure) was accurately weighed into a 100.0 mL volumetric flask, dissolved and volume was made up to 100.0 mL with water (1 mg/mL). 10.0 mL of this solution was further diluted to 50.0 mL with water (200 µg/mL).[11]

Determination of wavelength of maximum absorbance:
2.0 mL of working reference standard solution was pipetted into a 25.0 mL volumetric flask. 10.0 mL of resorcinol reagent was added and solution was cooled to room temperature. The solution mixture was heated in a boiling water bath for 1 hour and cooled to room temperature by placing in ice-cooled water. The volume was made up to 25.0 mL distilled water. The yellow-orange coloured solution was scanned in 400 - 800 nm range against reagent blank.[11]

Preparation of sample solution:
Sample of chondroitin sulphate (about 100 mg) was transferred to 100.0 mL volumetric flask, dissolved in about 50.0 mL of double distilled water, sonicated for 15 minutes and made up the volume with double distilled water. The resulting solution was filtered through Whatman® filter paper. Filtrate solution (10.0 mL) was diluted to 50.0 mL with distilled water. From this, a 2.0 mL was transferred into 25.0 mL volumetric flask, and added with 10.0 mL of resorcinol reagent solution. Solution was cooled to room temperature. The solution mixture was heated in a boiling water bath for 1 hour and cooled to room temperature by placing in ice-cooled water. The volume was made up to 25.0 mL with distilled water. The absorbance of yellow-orange colour chromogen was measured at 435 nm against reagent blank.[1]

Method validation:
The method was validated for linearity, accuracy, system precision, method precision, detection limit, quantitation limit and stability of the absorbance of yellow-orange colour chromogen analytical solution during assay.

Linearity:
Serial concentrations of chondroitin sulphate in range between 4-32 ppm were made from chondroitin sulphate standard stock solution.[11]

Accuracy:
Accuracy of the method was determined in terms of percent recovery of standard chondroitin sulphate at three different concentrations of 50%, 100% and 150%.[11] Results of the recovery study were found to be within the acceptable criteria of 80-110%, indicated sensitivity of themethod towards detection of chondroitin sulphate and non interference of excipients in the method.[12]

System precision:
The precision of the system was determined by 6 repetitive absorbance of the same standard solutions by using 2.0 mL of stock solution. The values of % RSD of system precision study were in within the acceptable limit. Hence the method provides good precision.[11]

Method precision:
The precision of the method for the assay of chondroitin sulphate was determined by the assay of six aliquots of the homogeneous sample. The values of % RSD of method precision study were in within the acceptable limit. Hence the method provides good precision and reproducibility.[11]

Detection limit:
The detection limit was determined by analyzing chondroitin sulphate with known concentrations and establishing minimum level at which the chondroitin sulphate was reliably detected. The detection limit (DL) may be expressed as; DL =3.3 σ/ S, where σ = the standard deviation of the responseS = the slope of the calibration curve.[11]

Quantitation limit:
The quantitation limit was determined by analyzing of chondroitin sulphate with known concentrations and establishing minimum level at which the chondroitin sulphate was quantified with acceptable accuracy and precision. The quantitation limit (QL) may be expressed as: QL=10 σ/ S, where σ = the standard deviation of the response and S = the slope of the calibration curve.[11]

Solution stability:
The stability of the analytical solution for assay of chondroitin sulphate was determined by measuring absorbance of sample solution at fixed intervals of time (every 1 minute for 15 minutes). The % RSD for the assay values for chondroitin sulphate should be up to 2%.[11]

RESULTS:

Isolation of chondroitin sulphate from chicken shanks:
The mean yields of chondoritin sulphate obtained from chicken shank in dry weight were 36.35 % and 16.30% from 8h and 6h incubation, respectively.

Characterization of Chondroitin Sulphate Fourier Transform – Infrared:

The FTIR spectra of chondroitin sulphate sample was overlayed with the spectra of chondroitin sulphate standard. The FT-IR spectra of chondroitin sulphate from shank cartilage was identical with the spectra of chondroitin sulphate standard in fingerprint area. The Overlay spectra of CS standard and CS from chicken shank (Figure 2).

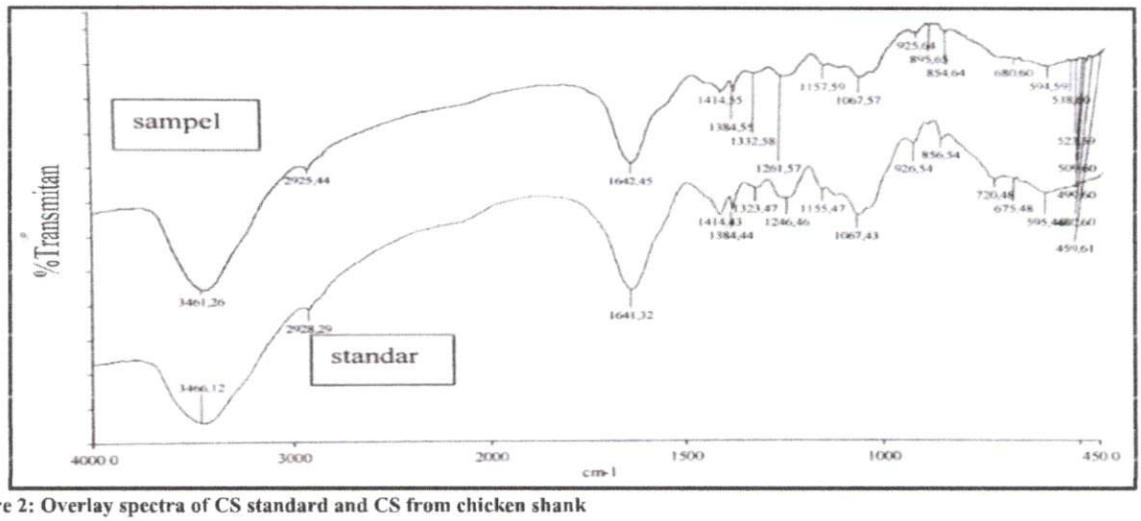


Figure 2: Overlay spectra of CS standard and CS from chicken shank

Overlay of the spectras showed that samples and standards have a similarity in the fingerprint area (wave number 1500-400 cm^{-1}) with the characteristic absorption at the wave number 854.64 cm^{-1} for sample and 856.54 cm^{-1} for standard chondroitin sulphate.[5] In addition there was absorption at wave number 1641.32 cm^{-1} for standard and 1642.45 cm^{-1} for the samples indicated the presence of a carbonyl group. There was also absorption at wave number 3466.12 cm^{-1} for standard and 3461.26 cm^{-1} for the samples indicated the presence of hydroxyl grou.[13]

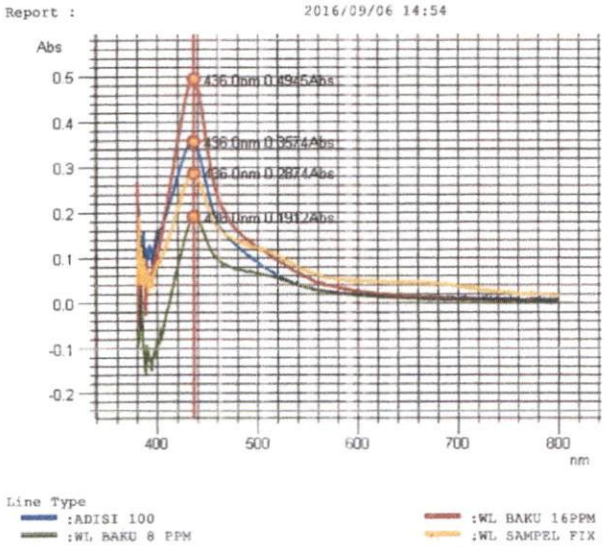


Figure 3. Overlay UV spectra of CS standard and CS from chicken shank.

SpectrophotometricMethod:

Determination of wavelength of maximum absorbance:

The wavelength of maximum absorbance of yellow-orange colour chromogen was found at 436 nm as shown in (Figure3).

Method validation:

Linearity:

The linearity of chondroitin sulphate using serial concentration of 4-32µg/mL showed r = 0.9954 and Vxo= 10.49 %.

Accuracy:

The accuracy of the method was established by recovery study, referring to AOAC guidelines, and the obtained % recovery were ranged from 82.48-108.88%. Results of the recovery study were found the acceptable criteria of 80-110%, indicating sensitivity of the method towards detection of chondroitin sulphate and non interference of excipients in the method.[12]

System precision:

The precision was good with % RSD was 0.6% and within the acceptable limit (less than 7.3%). Hence the method provided good precision.

Method precision:

The precision of the method for the assay of chondroitin sulphate was good with % RSD of 0.08%. This was within the acceptable limit (less than 7.3%). Hence the method provided good precision and reproducibility.

Detection limit and Quantitation limit:
The detection limit and quantification limit were found to be 0.15 ppm and 0.46 ppm, respectively.

Solution stability:
The % RSD for the absorbance values for chondroitin sulphate up to 15 minutes was 2.13%, indicating that the analytical solution was stable up to 15 minutes.

Determination of chondroitin sulphate in the sample:
Concentration of chondroitin sulphate in samples for 8h extraction was 87.87% and 53.74% for 6h extraction. Statistical analysis of the results shows that these two concentrations were significantly different.

DISCUSSIONS:

Chondroitin sulphate (CS) can be extracted from chicken shank obtained from farm wastes using acid hydrolysis extraction method. CS mean yields in dry weight obtained from 6h and 8h incubation were 16.30% and 36.35 %, respectively, whilst yield from chicken keel was reported 14.08% and from shark cartilage was 9.6% (dry weight).[5] The FT-IR spectra of CS from shank cartilage was identical with CS standard. Concentration of chondroitin sulphate in sample from 8h and 6h extraction were 87.87% and 53.74% respectively. CS purity from chicken shank cartilage was 87.87% for 8h extraction by (acid hydrolysis extraction method) whilst certificate of analysis of the CS from Fenchem Biotek Ltd stated 90.47% and those from Chemical Point UG Germany stated 90.7%. Therefore, the method needs more purification steps. Quantitative analysis and validation were conducted using spectrophotometric method were showed satisfactory results. The precision was good with RSD<7.3%. The accuration was good with percent recovery of 82.48-108.88%. The detection limit and quantification limit were 0.15 ppm and 0.46 ppm, respectively. This study showed that chicken shank cartilage is a readily available source of chondroitin sulphate.

CONCLUSION:

The present work showed that chicken shank cartilage is potential source of chondroitin sulphate. The yield was 36.35 % (dry weight) and the purity was 87.87% indicated a feasible source for isolation of chondroitinsulphate.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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14

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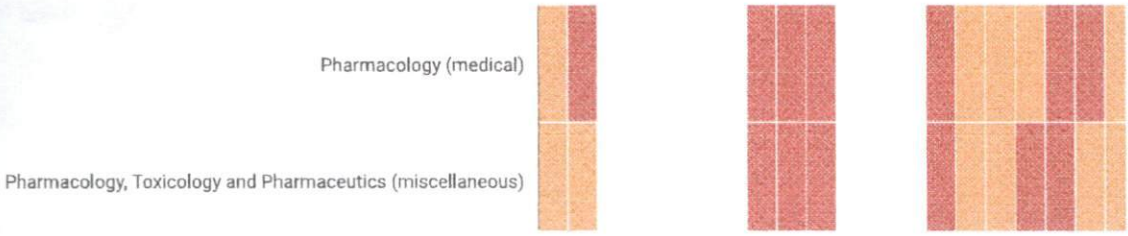

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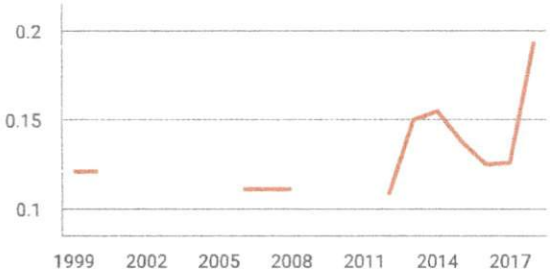

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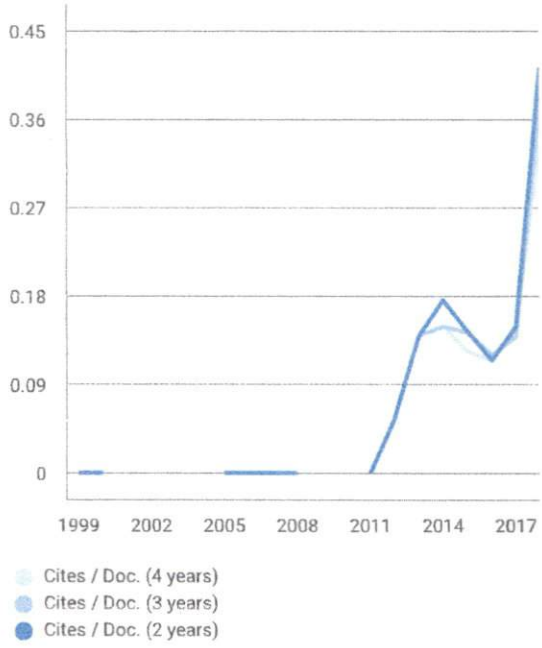

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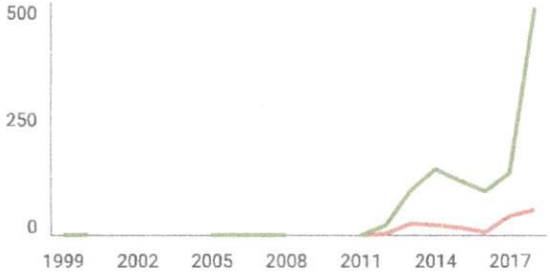

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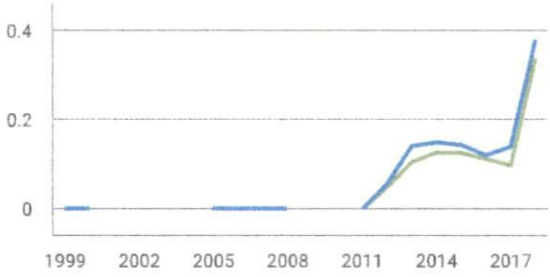

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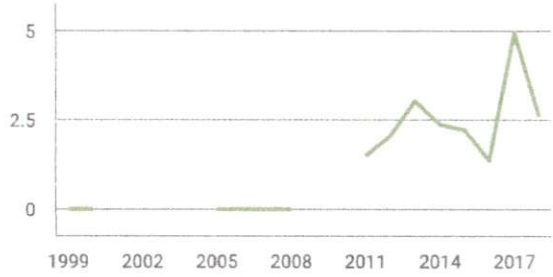

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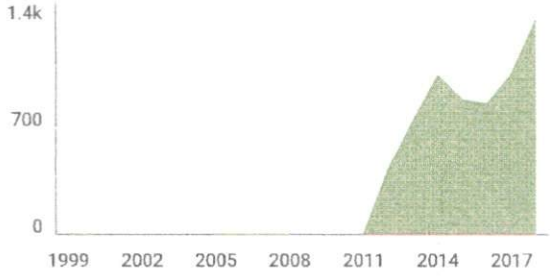

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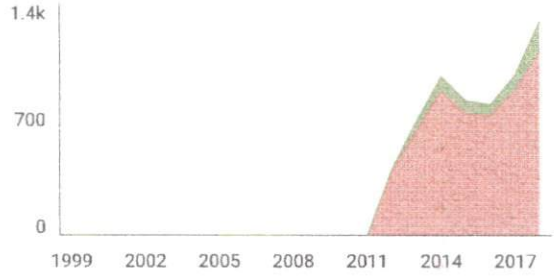

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