MATERIALS AND METHODS

Collection of plant materials

The shells of *Cocos nucifera* from Arecaceae family were collected in Thiruvallur district, Tamilnadu, India and the shells of *Anacardium occidentale* from the Anacardiaceae family were collected from Panruti, Cuddalure district, Tamilnadu, India.

Preparation of shells for oil

The collected shells were cleaned and sun dried. The shells were polished with sand paper to smoothen the shell's surface. Further, the smoothened shells were ground to small pieces to make extraction of crude Coconut Shell Oil (CSO) and Cashew Nut Shell Oil (CNSO).

Extraction of Coconut and Cashewnut shell oils

For the extraction of CSO and CNSO, the most common method of commercial extraction of pyrolysis was used. The grounded shells (250gms) were heated in an earthern pot at 200^{o}C to 250^{o}C temperature for a span of 3h. The outer part of the shells burst open and release oil. This method yields 25cc of oil from the shells.

Fractionation of shell oil using organic solvents

CSO and CNSO were extracted with the organic solvents namely ethanol, chloroform, acetone, petroleum ether and aqueous extracts in 1:3 v/v ratio. After this process it was concentrated by vacuum evaporator and the obtained residue was considered as crude extracts. These crude extracts were labeled and stored for further research. Percentage yield of the extract was calculated.

PHYTOCHEMICAL ANALYSIS

The phytochemical screening of compounds was performed in CSO and CNSO using the standard procedures described by Harborne (1973) as described below and the results were recorded.

Detection of Alkaloids by Wagner's Test

About 2 ml of extract dissolved in dilute HCl and treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of Brown or reddish precipitate indicates the presence of alkaloids (Tiwari *et al*., 2011).

Detection of Carbohydrates by Benedict's Test

Treated 2 ml of extract with Benedict's reagent and gently heated. The development of orange red precipitate indicates the presence of reducing sugars (Tiwari *et al*., 2011).

Detection of Saponins by foam Test

About 2 ml of sample was mixed with 2 ml of sterile distilled water and shaken vigorously for about 30 seconds. It was allowed to stand for 10 minutes, if foam observed and persists indicates the presence of saponins (Tiwari *et al*., 2011).

Detection of Phenols by ferric chloride Test

Each sample was treated with 3-4 drops of Ferric chloride solution. The presence of phenols indicated by the formation of bluish black colour (Mishra *et al*., 2011).

Detection of Tannins by ferric chloride test

Sample diluted with water and 3-4 drops of 10% Ferric chloride solution was added. A blue colouration in extract indicates the presence of tannins (Mishra *et al*., 2011).

Detection of Flavonoids by Lead acetate test

About 1 ml of sample was treated with drops of lead acetate solution and yellow colour was observed for the presence of flavonoids (Tiwari *et al*., 2011).

Detection of Amino acids by Ninhydrin Test

To 1 ml of sample was mixed 0.25% ninhydrin reagent and boiled for few minutes. A blue colouration indicates the presence of aminoacids (Mishra *et al*., 2011; Heroaurt *et al*., 1988).

Detection of Diterpenes by copper acetate Test

Samples were dissolved in water and 3 - 4 drops of Copper acetate solution was added. The presence of emerald green confirms the presence of diterpenes (Obasi *et al*., 2010).

Detection of Glycosides modified Borntrager's Test

Glycosides were detected by adding 5 ml of diluted Sulphuric acid with 5 ml of extract and boiled for 15 minutes. After boiling it was cooled and neutralized with 10% NaOH, and then 5 ml of Fehling solution was added. Formation of brick red precipitate was observed to confirm the presence of glycosides (Mishra *et al*., 2011).

Detection of Quinones by conc. H_2SO_4 Test

The sample (1ml) was mixed with 1ml of conc. H_2SO_4. The presence of quinones indicated by the formation of red colour.

Detection of Terpenoids by Salkowski's Test

About 5ml of sample was mixed with 2 ml of chloroform and 3 ml of conc. H_2SO_4 added carefully to form a layer. A reddish brown colouration at the interface was observed which indicates the presence of terpenoids.

Detection of Proteins by Biuret Test

Five ml of sample was treated with Biuret reagent. Pink colour was appeared which confirm the presence of proteins (Khanam *et al*., 2013).

Detection of Steroids by Harbourne's Test

Steroids were identified when 2 ml of acetic anhydride was added to 0.5 ml of sample with 2 ml of H_2SO_4. The colour changed from violet to blue that indicate the presence of steroids.

Detection of Cardiac glycosides by Kellerkiliani Test

Sample (200 µl) was treated with 100 µl of glacial acetic acid containing one drop of Ferric chloride solution, followed by adding 1 ml of conc. Sulphuric acid. Presence of Deoxyribose sugar characteristics of cardenolides was indicated by a brown ring found in the interface region (Godghate *et al*., 2012).

Detection of Oxalate by glacial ethanoic acid Test

About 3 ml of sample was treated with few drops of glacial ethanoic acid. The presence of oxalate confirmed by the greenish black colouration (Charles *et al*., 2013).

Detection of Anthocyanin by HCl and NH_3 Test

Sample of about 2 ml was mixed with 2 ml 2N HCl and Ammonia. The change of colour from pink red to blue violet indicates the presence of anthocyanin compound (Godghate *et al*., 2012).

Detection of Leucoanthocyanin by isoamyl alchohol

About 5 ml of isoamyl alchohol treated with 5 ml of sample. The presence of leucoanthocyanin indicated by the appearance of red colour in the upper layer (Godghate *et al*., 2012; Kokate *et al*., 2006).

Detection of Coumarin by Mace method

Each sample (1ml) was mixed with 1ml of 10% NaOH. Formation of yellow colour confirmed the presence of coumarin (Kumar *et al*., 2013).

Detection of Carboxylic acid by Sodium bicarbonate test

Few ml of Sodium bicarbonate solution was mixed with 1ml of sample. Formation of effervescence occurs due to the liberation of Carbon dioxide that indicates the presence of carboxylic acid (Kumar *et al*., 2013).

Detection of Xanthoproteins by HNO_3 and NH_3 Test

One ml of sample was mixed with few drops of conc. HNO_3 and NH_3 solution. The presence of xanthoproteins indicated by the formation of reddish orange precipitate (Kumar *et al*., 2013).

BIOLOGICAL ACTIVITY

ANTIMICROBIAL ACTIVITY

About 10 ml of sample was extracted with 30 ml of the organic solvents namely ethanol, chloroform, acetone, petroleum ether and aqueous separately. After extraction, the extract was concentrated by using vacuum evaporator and the obtained residue were stored as crude extracts, labeled and kept at 4^{o}C for further research.

Preparation of medium

The antibacterial activity was studied by using Muller Hinton Agar medium (MHA) and the antifungal activity was studied by using Potato Dextrose Agar medium (PDA).

Microbial strains and preparation of inoculum

The bacterial strains used in this study were two Gram positive bacteria such as *Staphylococcus aureus, Enterococcus faecalis* and four Gram negative bacteria namely *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella paratyphii*. Bacterial stock cultures were maintained on Muller Hinton Agar (MHA) medium at 4^{o}C. Active bacterial culture was prepared by taking a loopful of cells from stock culture and inoculated into fresh Nutrient medium kept at 37^{o}C overnight. To obtain desirable cell counts and for bioassays, bacterial cells that was overnight grown was subcultured in MHA at 37^{o}C.

The fungal pathogens namely *Epidermophyton floccosum, Aspergillus niger, Penicillium, Microsporum, Candida albicans* and *Aspergillus flavus* were used for the antifungal study. Fungal cultures were maintained on Potato Dextrose agar slants and stored in the refrigerator. Active fungal culture was prepared by inoculating from stock culture into a fresh medium and inoculated at 24^{o}C for 48 h. The fungal culture was subcultured on PDA slants.

Antibacterial activity

The well diffusion method was used to evaluate antibacterial activity using Muller Hinton Agar (MHA) medium. The prepared medium was autoclaved for 15 minutes at 121^{o}C, at 15 lbs pressure. The sterilized MHA medium was poured into sterile Petri plates and allowed to solidify. After solidification of the medium, the 24 h bacterial culture was inoculated using sterile cotton swabs dipped in bacterial suspension. Sterile 5 mm cork borer was used to make agar wells. In each Petri plate five wells were made and extracts were added as 30µg/µl, 40µg/µl and 50µg/µl solutions. DMSO was used as control and tetracycline 30 µg as positive control. The plates were labeled and incubated at 37^{o}C. The zone of inhibition (MI) was measured after 24 h and the results were tabulated.

Antifungal activity

To screen the antifungal activity the spore suspension method was used in agar well diffusion assay. Petri plates were prepared by pouring 15-20 ml of molten PDA medium and allowed to solidify and test fungal cultures were spread evenly using sterile swab uniformly and allowed to dry for 10 min. With the help of sterile cork borer wells were made. Specific amount of samples at different concentrations of 30 µg/µl, 40 µg/µl and 50 µg/µl were added into the wells and left for 30 min. at room temperature for the diffusion of compounds. Ketoconazole used as positive control. The plates were kept for incubation at 28^{o}C for three to six days. Inhibition zones were recorded in millimeters and the results were tabulated.

MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC of acetone, ethanol and petroleum ether extracts was determined by broth microdilution method as per the procedure described by Eloff (1998). In a titre

plate 12 wells were filled with 0.5ml sterilized Mueller Hinton Broth medium. Well 1 served as growth control, well 12 as antibiotic control. Sequentially, wells 2-11 were added with 0.5 ml of a mixture of culture medium using sterile micropipette. Tetracycline Hydrochloride (0.1mg/mL) was used as control for the *S. aureus*. The samples were serially diluted to a concentration sequence from 150µg/µl to 0.15µg/µl and incubated at 37^0C for 24h. The lowest concentration (highest dilution) of the extract that produced no visible growth (no turbidity) in the first 24 h was identified and that was compared with the control wells and considered as initial MIC. The resulting turbidity was observed by optical density readings at 660nm with a Beckman DU-70 UV-Vis Spectrophotometer. Three repetitions were made and the results were tabulated.

ANTIOXIDANT ACTIVITY

2,2-diphenyl-1-picryl hydrazyl radical scavenging (DPPH) Assay

The Antioxidant activity of the samples were evaluated on the basis of free radical scavenging assay; measured *in vitro* by using the stable compound of 2,2-diphenyl-1-picryl hydrazyl based on the method of Wang *et al*.,(2011); As per reference Bhuiyan *et al*., (2009) was used. About 1 mL of 0.1mM solution of DPPH in methanol was added to samples (2.5 mL) of different concentrations (100µg/µl to 500µg/µl) and 1.0 mL of DPPH and 1.0 mL of methanol were used as control. The reaction mixture was kept in the dark at room temperature for 20 minutes. After incubation the optical density was measured using spectrophotometer at 517 nm. BHT was used as standard. The free radical scavenging assay was calculated according to the formula:

$$\text{DPPH scavenging activity} = \frac{(Ac - As)}{Ac} \times 100$$

Where Ac = absorbance of the control

As = absorbance of the extract

The IC_{50} (Inhibitory Concentration – 50 %) values were evaluated by plotting x, y scatter trend line with regressive equation.

Nitric Oxide scavenging Assay

Sodium nitroprusside generates nitric oxide and is measured by Greiss reaction. About 3.0 mL of 10 mM Sodium nitroprusside in phosphate buffer (0.025m, pH 7.4) was treated with 2.0 mL of samples at different concentrations of 100µg/µl to 500 µg/µl. Incubate the resulting solutions at 25ºC for 1h. Same procedure was repeated with methanol as blank, which serve as control. Curcumin, caffeic acid used as positive control. After the incubation period, 5.0 ml of the Griess reagent was added with the samples and the absorbance was recorded at 546 nm. Griess reagent prepared by adding 1% sulphanilamide, 0.1% naphthyethylene diamine dihydrochloride in 2% H_3PO_4. Ascorbic acid was used as standard. The Nitric oxide scavenging assay was calculated by comparing absorbance values of control and sample values.

ANTICANCER ACTIVITY

Cell line and culture

Breast cancer cell line (MCF-7) was obtained from National Centre for Cell Sciences (NCCS), Pune. The collected cells were maintained in DMEM- High glucose, supplemented with 10% FBS, Penicillin (100U/ml) and streptomycin (100µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37ºC.

Reagents for Cell line

DMEM was purchased from Himedia laboratories; Fetal Bovine Serum (FBS) was purchased from Cistron laboratories. Dimethyl sulfoxide (DMSO) and Methyl thiazolyl Diphenyl tetrazolium bromide were bought from Sisco Research laboratory chemicals, Mumbai.

***Invitro* assay for cytotoxicity activity (MTT assay)**

The cytotoxicity effect of the samples was evaluated by MTT assay (Moseman, 1983). The cells (1 x 10^5 / well) were cultured into the plates in 24 well plates and incubated overnight at 37ºC for 24 h with 5% CO_2 condition. After the cell reaches the confluence, different concentrations of the samples were added and incubated for 24 h. At the end of the treatment, the sample was washed with

Phosphate Buffered Saline (PBS) at pH 7.4. After this wash 100µl/well (5mg/mL) of 0.5% 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 h. After incubation period, 1ml of DMSO was added to all the wells. The absorbance was measured at 570 nm with UV spectrophotometer using DMSO as the blank. Measurement was performed to evaluate the effect of the samples on the MCF -7 proliferated cells. The concentration required for 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells × 100

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) FINGERPRINT ANALYSIS

Preparation and application of samples

The ethanol extract of CSO and CNSO samples were separately made upto 10 mL in a standard flask and applied on the plates. The application of samples to the plates in HPTLC separate the volatile and nonvolatile compounds. HPTLC studies were carried out as per the procedures given by Wagner *et al* (1996).

About 0.5µl of the sample and 3 µl of standard solution were loaded as band length of about 5 mm separately in TLC aluminium sheets precoated with silica gel 60 F_{254} 20 x10 cm with 0.2 mm µm thickness (E.Merck, Darmstadt, Germany). The plates were prewashed with ethanol and activated for 5 min. at 60^{o}C. Application rate was constant and employed as 0.1µl $s^{-1}$with 6 mm bandwidth. The dimension of slit was about 6.0 mm and scanning speed was 10 mm s^{-1}. The aluminium plates were attached to CAMAG HPTLC system programmed through WIN CATS software. After the application of the spots, the plates were developed in twin trough glass chamber. A TLC scanner with win CATS software was used for scanning the TLC plates.

Development of solvent system

Various solvent systems were used to determinate HPTLC fingerprint analysis for various secondary metabolite groups like alkaloids, glycosides, terpenoids,

flavonoids and saponins separately. The mobile phase consisted of Toluene: Ethyl acetate: Glacial Acetic acid (9:1:0.2) (v/v). 15ml of mobile phase was utilized per plate. For mobile phase the optimized chamber saturation time was 15 min. at room temperature of 25^{o} C ± 2 at relative humidity of 60% ± 5.

Development of chromatogram

The chromatogram was developed in twin tough glass chamber saturated with the solvent system Toluene : ethyl acetate : Glacial acetic acid (9:1:0.2). The monochromatic bandwidth was developed at 20 nm; the developed chromatogram length was 8 cm. The loaded plates were allowed to dry in the air and scanned under remission mode at UV-254 nm, UV-366 nm and visible light by densitometric scanner after derivatised using Vanillin – Sulphuric (VS) acid.

Scanning and Detection of spots

The chromatographic plates after development of bands were dipped in derivatization reagent namely Dragendroff reagent and dried again for 10 min on hot mode. After drying process the plates were heated for 15 min. at 100^{o}C in hot air oven. The plates were scanned in a photo documentation chamber (CAMAG REPROSTAR 3) and the images were captured in visible light, UV 254 nm and UV 366 nm. The spots and peaks of the compounds were detected and their R_f values and peak areas with retention time were recorded by WIN CATS software.

$$\text{Retention factor } (R_f) = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

GCMS ANALYSIS (GAS CHROMATOGRAPHY AND MASS SPECTROPHOTOMETRY)

The phytochemical search of ethanolic extract of samples was carried out on GC Clarus 500 Perkin Elmer system interfaced to a mass spectrometer instrument. The software employed for GCMS analysis was Turbomass ver 5.2 column Elite 5ms fused with the silica capillary column (30 x 0.25 mm ID x 0.25µm film thickness, 5% Phenyl, 95% Dimethyl Polysiloxane). Electron impact mode operated for GCMS detection was 70 eV. Ionization energy, Helium gas (99.999%) was utilized as the

carrier gas at 1mL/min of constant flow rate with injector temperature of 290^{o}C. Occurrence of Electron ionization and the ion source temperature was 150^{o}C. In the gas chromatography the temperature was programmed from 50^{o}C increased to 220^{o}C at 2^{o}C/min hold for 10min; From 220^{o}C to 280^{o}C with 4^{o}C/min hold for 10 min. mass spectra was recorded at 70 eV. Total GC running time was 36 min. For each compound the relative percentage was compared and calculated with its average peak area to the total area.

Identification of phytocompounds

Identification of the phytocompounds from GCMS data was carried out from National Institute of Standard and Technology (NIST Version – Year 2005) and Wiley Spectral library search programme has more than 62,000 patterns. Identification of mass spectrum of the unknown compounds was done by comparing it with the spectrum of the known compounds which were well stored in the databases. The name of the molecules, molecular formula, molecular weight, 2D structure of the compounds and their biological activity of the compounds were determined.

IN- SILICO MOLECULAR DOCKING STUDIES

Virtual screening is a computational technique utilized to screen potential compounds against specific target proteins from the chemical compound libraries. Structural Based Virtual Screening (SBVS) was used to find novel and potential lead compounds. SBVS efficiently estimates a ligand to bind to the target proteins with high binding energies. In the present study, the drug discovery platform Mcule was used to employ SBVS (Kiss *et al*., 2012; Trott and Olson, 2010). For SBVS, each ligand was docked to the binding site of HER2, EGFR and HSP90-α and scored by maximum binding affinity.

Molecular docking against Human Epidermal growth factor Receptor 2

Human epidermal growth factor receptor 2 (HER2) is one of the major targets against which breast cancer treatment and other carcinomas were treated (Mirzaie *et al*., 2013). HER2 receptor plays a key role in the cell growth and differentiation

process, an over expression to develop carcinomas. HER2 is over expressed in 15–30% of invasive breast cancer development (Hynes and Stern 1994).

Catalytic activity

ATP + a [protein]-L-tyrosine = ADP + a [protein]-L-tyrosine phosphate.

The crystal strucure of HER2 (3PP0) was resolved by Aertgeerts *et al* (2011) and available in Protein Data Bank (https://www.rcsb.org/structure/3pp0) (Resolution: 2.25A^{o}) was used for molecular docking.

Crystal strucutre of HER 2 (3PP0) was obtained from Protein Data Bank for molecular docking (Mirzaie *et al*., 2013). Molecular docking was performed using Autodock and Autodock Vina in the PyRx platform. PyRx (0.8) version was used for the docking study (http://pyrx.sourceforge.net). Binding affinity in terms of energy value (kcal/mol) of research molecules with HER2 protein was compared with 2-{2-[4-({5-chloro-6-[3-(trifluoromethyl)phenoxy] pyridin-3-yl}amino)-5H–pyrrolo[3,2-d] pyrimidin -5-yl]ethoxy}ethanol as the inhibitor which is crystallized with the complex structure 3PP0.

Molecular docking against Epidermal growth factor receptor (EGFR)

The FDA approved drugs for breast cancer treatment has various adverse side effects. Therefore, it becomes important to discover new drugs for breast cancer treatment. Epidermal growth factor receptor (EGFR) is another important drug target for breast cancer treatment. EGFR is a cell surface receptor. EGFR family is activated, leading to scattering and invasion of breast epithelial cells. Therefore EGFR is an attractive drug target for breast cancer treatment (Yousuf *et al*., 2017).

The crystal strucure of Epidermal growth factor receptor (1M17) was resolved by Stamos *et al*., (2002) and available in Protein Data Bank (Resolution: 2.6A^{o}) was used for molecular docking (https://www.rcsb.org/structure/1m17). Molecular docking was performed using Autodock and Autodock Vina in the PyRx platform. PyRx (0.8) version was used for the docking study (http://pyrx.sourceforge.net). Binding affinity in terms of energy value (kcal/mol) of research molecules with EGFR protein compared with [6,7-bis(2-methoxy-ethoxy)quinazoline-4-yl]-(3-

ethynylphenyl)amine as the inhibitor which is crystallized with the complex structure 1M17.

Molecular docking against Heat shock protein HSP 90-α

Heat shock protein HSP 90-alpha (HSP90-α) is another target for breast cancer treatment. HSP90-α protein plays a crucial role in cellular protein folding. It also involves programmed cell death and cell senescence during hyperthermia. It is an essential protein for the cell survival. Therefore HSP90 was also selected for docking study. The crystal strucure of Heat shock protein HSP 90-alpha (HSP90-α) was resolved by Ying available in Protein Data Bank (https://www.rcsb.org/structure/3tuh) (Resolution: 1.8A^{o}) was used for moleculare docking. Crystal strucutre of Heat shock protein HSP 90-alpha (HSP90-α) (3TUH) was obtained from Protein Data Bank for molecular docking (Yousuf *et al.*, 2017). Molecular docking was performed using Autodock and Autodock Vina in the PyRx platform. PyRx (0.8) version was used for the docking study (http://pyrx.sourceforge.net). Binding affinity in terms of energy value (kcal/mol) of research molecules with HSP90 protein was compared with 5-[2,4-dihydroxy-5- (propan-2-yl) phenyl]-4-(1-methyl-1H-indol-5-yl)-2,4-dihydro3H 1,2,4 -triazol-3-one as the inhibitor which is crystallized with the complex structure 3TUH.

BIOFUEL

The samples CSO and CNSO were filtered for the removal of the dirt particles. This process generally carried out by warming up the liquid. After warming up the samples the lye was determined. This process is the most crucial and the most important stage in the preparation of biofuel. To speed up the reaction process it is necessary to remove the water content from the samples. The samples were heated to 100^{o}C for 10 min and the water within the samples were evaporated (Boocock *et al*., 1996, Efavi *et al*, 2018)

Preparation of Sodium methoxide

In this stage methanol is treated with sodium hydroxide to yield sodium methoxide. Generally 20% of methanol was used with the total quantity of the samples. The obtained residue was heated between 110^{o}C to 130^{o}C. After the heating

process the sample was mixed thoroughly and carefully without splashing of the liquid. The mixed samples were allowed to cool down (Zhou and Boocock, 2006).

Separation of biofuel

Biofuel produced by adding 200 mL of extracted oil, 40 mL methanol and 1g NaOH. At the first step of the experiment methanol was mixed with NaOH stirred until fully dissolved. Then 200 mL of CSO and CNSO extracts were heated separately until 55-60^{o}C. After reaching the desirable temperature the methanol and NaOH mixture was continuously mixed with the heated oil extracts and the reaction was carried out for 1h and cooled. After cooling the biofuel float at the surface whereas the heavier particles like glycerin separated at lower layers. The lower layer was separated and removed. The remaining liquid was biofuel and used for various purposes. For washing process, 50% of water was mixed to the biofuel in order to extract the contaminants. The mix was separated by forming a top biofuel layer and a bottom aqueous layer due to the density and immiscibility. If the separation was completed the aqueous layer was removed and the process repeated until the contaminants were removed completely. The next step was the drying process made by heating the biofuel sample to a temperature range of 105 to 110^{o}C. It was important that the temperature should not be less than 105^{o}C and more than 110^{o}C. Biofuel was heated for about 30 min. until all the water contents evaporated and separation of sample was completed (Moser, 2009; Jaber *et al*., 2015).

Kinematic Viscosity

Kinematic viscosity is a measure of the resistance to flow of a fluid and equal to the absolute viscosity divided by its density. Procedures for measuring the viscosity in the laboratory was done by the traditional method defined by Indian Standard Methods of test for petroleum and its products (IS:1448 Part 25, 1976). Biofuel viscosity was measured by using a Brookfield Viscometer model DV-1-4644. It consists of a set of seven spindles with accuracy ± 1%. The spindles measure viscosity range from 1 to 13300000 cP. The CSO and CNSO sample (10 mL) was applied in viscometer water bath for 30 min. The viscosity of the biofuel and the timings were noted and calculations were made.

cSt = cP / SG

cSt = Kinematic viscosity

cP = absolute viscosity

SG = fluids specific gravity

Viscosity at 40^{o}C constant value is 0.27885 cst

Viscosity at 50^{o}C constant value is 0.27885 – 0.0026 = 0.27625 cst

Viscosity at 100^{o}C constant value is 0.27885 x 0.992 = 0.266192 cst

Standard calculation

W = M x h – (CVt + CVw) / T

W = Water

M = weight of sample

H = constant value – 6321 (benzoic acid calorific value)

CVt = calorific value in thread – 21

CVw = calorific value in wire – 9.32

T = rising temperature.

Flash point

The flash point is the temperature in which the vapour found above the fuel reaches the lower flammability limit and ignite under a given set of test conditions specified in ASTM D9. Samples with a flash point below 130^{o}C were tested for methanol content by EN 14110. The flash point of biofuel was measured by flash point tester which consists of 100mL closed copper cup, heater and a source that gives continuous sparks connected to small engine, disporator coil. The engine was used to rotate the disporator, which was used to fractionate the current to electrical pulses. Biofuel sample was heated and the vapour accumulated inside the cup, that time was sufficient to ignite the flash light and the temperature was recorded and known as end point of a flash point.

Density

To calculate the density of CSO and CNSO biofuel extracts, three masses was measured; mass of empty pycnometer, mass of pycnometer with ultra pure water, mass of pycnometer with biofuel samples. Temperature was measured at the sametime for ultra pure water and biofuel samples. Standard conical flask (25mL) was washed and dried in hot air oven, then cooled in dessicator and mass of the

empty weight was taken. After adding 25mL of biofuel samples and the mass pycnometer was recorded and the density of the samples calculated using the following formula,

$$P = m / V$$

Where

P = density

m = mass

V = volume

Gross Calorific value (GCV)

The GCV of a fuel at a constant volume was the number of heat units which would be liberated when unit of fuel weight was burnt at constant volume in oxygen saturated with water vapour at 15^{o}C, the residual products are CO_2, SO_2, N and H_2O, the water content found at vapour, being in the liquid form. The calorific value was closely related to the density of the liquid which was easily determined by using a hydrometer. The relationship affords the most important means of computing the calorific value of petroleum and its products. Total heat of combustion and constant volume and specific gravity of the samples were calculated by the following formula,

$$q_v = 12400 - 2\ 100\ d^2$$

Where q_v = calorific value at constant volume in cal/g

d = specific gravity at 15.56^{o}C.

The calorific value at constant pressure is given by

$q_p = q_v - H\ (WL-C)$

q_p = calorific value at constant pressure, in cal/g

qv = calorific value at constant volume in cal/g

H = Hydrocarbon content

d = specific gravity

W = No. of grams of water formed from ig of hydrogen

L = latent heat of vapourization of water at 20oC

C = change in volume from beginning to the final

Cetane number

The cetane number (CN) is a most important property of diesel oils indicating the ignition quality of fuels (the higher, the better), somewhat analogues to octanes for gasoline. ASTM published an updated ASTM D7170 test procedure for derived CN using the ignition delay principle that provides for an economical and easy way to determine the diesel fuel cetane number.

Methods for measuring CN of biofuels

There are basically four different methods used in the determination of CN (Barsamian, 2009).

a) Use of a cetane engine
b) Use of an Ignition Quality Tester
c) Use of a Near Infra Red (NIR) Analyzer
d) Use of Cetane devices

a) Use of Cetane engine:

This is the common standard used as referee method (ASTM D613); measurement was reliable and straightforward once the engine was set up and calibrated.

b) Use of an Ignition Quality Tester (IQT)

In this method the time delay measures between the start of fuel injection and the start of significant combustion through auto ignition of a pre-measured amount of diesel in a constant volume chamber. The time delay was used with a formula to calculate the Derived Cetane Number (DCN) which correlates to the D613 cetane engine. This was a relatively lower cost (less than one third of an engine), very reliable, high accuracy, faster, easily operated, and maintainable device. There are two ASTM test methods using IQT: D6890 and D7170. The difference between them was the number of cycles run for the test (32 cycles for D6890 Vs. 25 cycles for D7170) and the averaging of results.

c) Use of a Near Infra-Red (NIR) Analyzer

This method uses a Near Infra Red (NIR) analyzer to obtain the absorption spectrum of diesel which then was fed to a chemometric model to estimate cetane. Its reproducibility was an order of magnitude better than an engine; the reproducibility was the same (because we use the engine as the referee). It was very reliable and low maintenance if the chemometric property prediction model was set-up in accordance with ASTM E1655 practice and run as per ASTM D6122. The cost was the same as a cetane engine, but measures 10 to 15 diesel properties simultaneously every 1 to 2 minutes.

d) Use of Cetane Indices

This was an estimation of measured cetane number based on formulas in ASTM D976 or ASTM D4737. The calculations on diesel fuels T10, T50, T90 distillation points and gravity/density of samples was used to estimate the cetane number.